# ANTISENSE OLIGONUCLEOTIDE MODULATION OF HUMAN SERINE/THREONINE PROTEIN PHOSPHATASE GENE EXPRESSION

## INTRODUCTION

This application is a continuation-in-part of U.S. Application Serial No. 09/825,497, filed April 3, 2001, which is a continuation of U.S. Application Serial No. 09/371,252, filed August 10, 1999 (abandoned), which is a divisional of U.S. Application Serial No. 08/975,211, filed November 20, 1997 (U.S. Patent No. 5,948,202). The entire disclosures of these priority applications are incorporated herein by reference. This invention was made, in part, with Government support under grant NIH CA60750, awarded by the National Institutes of Health. The government may have certain rights in the invention.

## FIELD OF THE INVENTION

This invention relates to compositions and methods for modulating expression of human serine/threonine protein phosphatases (PPs), naturally present cellular enzymes which have been implicated in abnormal cell proliferation, carcinogenesis and tumor formation. Compositions and methods for specifically modulating the expression of serine/threonine protein phosphatase 1γ1 (PP1γ1), serine/threonine protein phosphatase 4 (PP4) and serine/threonine protein phosphatase 5 (PP5) are provided. This invention is also directed to methods for inhibiting hyperproliferation of cells; these methods can be used diagnostically or therapeutically. Furthermore, this invention is directed to treatment of conditions, particularly hyperproliferative conditions such as cancer, which are associated with expression of human serine/threonine protein phosphatases.

**BACKGROUND OF THE INVENTION**

Throughout this application various publications are referenced. The disclosures of each of these publications in their entireties are hereby incorporated by reference in this application.

The reversible phosphorylation of proteins on serine and threonine residues is a major intracellular control mechanism. Cell proliferation, cell signaling, gene expression and mitosis are among the cellular functions regulated by this mechanism. The phosphorylation state of a protein is controlled by kinases, which phosphorylate proteins, and phosphatases, which dephosphorylate proteins. A number of families and types of protein phosphatases exist, including tyrosine phosphatases and serine/threonine protein phosphatases (PPs). An increase in expression of certain PPs has been described in several tumor types. Modulation of expression of one or more serine/threonine protein phosphatases is desired for research, diagnostic, and therapeutic uses.

Small molecule inhibitors of protein phosphatases have been used to study PP function. The best characterized of these is okadaic acid, which is the causative agent of diarrhetic shellfish poisoning. It is a potent inhibitor of PP2A and PP1 and a much (roughly a thousand-fold) less potent inhibitor of PP2B. In spite of this difference in sensitivity, okadaic acid cannot easily be used to discriminate between PP1 and PP2A in cells. Other inhibitors of one or more PPs include tautomycin, cyclosporin A, dinophysistoxin, calyculin, microcystin, nodularin and cantharidin. Cairns et al., 1994, J. Biol. Chem. 269:9176-9183; Wera and Hemmings, 1995, Biochem. J. 311:17-29.

Improved inhibitors of protein phosphatases are desired for therapeutic, diagnostic and research uses.

Specific inhibitors of particular PP isoforms are particularly desired.

Oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. For example, workers in the field have now identified antisense, triplex and other oligonucleotide compositions which are capable of modulating expression of genes implicated in viral, fungal and metabolic diseases.

Antisense oligonucleotides have been safely administered to humans and clinical trials of several antisense oligonucleotide drugs, targeted both to viral and cellular gene products, are presently underway. For example, the oligonucleotide drug fomivirsen (ISIS 2922), is used to treat cytomegalovirus retinitis in AIDS patients. Another oligonucleotide drug, ISIS 2302, has been shown to be effective in Crohn's disease, a form of inflammatory bowel disease. It is thus established that oligonucleotides can be useful therapeutic instrumentalities and can be useful in treatment of cells and animal subjects, especially humans.

**SUMMARY OF THE INVENTION**

The present invention provides oligonucleotides which are targeted to nucleic acids encoding human serine/threonine protein phosphatases, particularly PP1γ1, PP4 and PP5, and which are capable of inhibiting PP expression. The oligonucleotides of the invention are believed to be useful both diagnostically and therapeutically, and are believed to be particularly useful in the methods of the present invention. The oligonucleotides are also believed to be useful as research reagents.

The present invention also comprises methods of inhibiting the expression of human PP. These methods are

believed to be useful both therapeutically and diagnostically as a consequence of the association between PP expression and hyperproliferation, particularly certain tumor types described hereinabove. These methods are also useful as tools, for example for detecting and determining the role of PP expression in various cell functions and physiological processes and conditions and for diagnosing conditions associated with PP expression. The methods provided are particularly useful for distinguishing between particular PP isoforms.

The present invention also comprises methods of inhibiting hyperproliferation of cells using oligonucleotides of the invention. These methods are believed to be useful in diagnosis, prevention and treatment of PP-associated cell hyperproliferation. These methods employ the oligonucleotides of the invention.

**DETAILED DESCRIPTION OF THE INVENTION**

The reversible phosphorylation of proteins on serine and threonine residues is a major intracellular control mechanism. Cell proliferation, cell signaling, gene expression and mitosis are among the cellular functions regulated by this mechanism. The phosphorylation state of a protein is controlled by kinases, which phosphorylate proteins, and phosphatases, which dephosphorylate proteins. A number of families and types of protein phosphatases exist, including tyrosine phosphatases and serine/threonine protein phosphatases (PPs). Antisense inhibitors of serine/threonine PPs are the subject of the present invention. Various types and isoforms of PPs have been described. These include PP1 (including $\alpha$, $\beta$, $\gamma 1$, $\gamma 2$ and $\delta$ isoforms) PP2A(including $\alpha$ and $\beta$), PP2B (including $\alpha$, $\beta$ and $\gamma$) (also called calcineurin-CNA, CMP), PP2C, PP4 (also called PPX), PP5 and PP6 (also called PPV and sit-4). For a

review of serine/threonine phosphatases and their nomenclature, see Cohen, P.T.W. (1997) Trends in Biol. Sci., 22:245-251. A selective increase in expression of PP1α and PP1γ1 has been described in liposarcoma and of PP1γ1 in osteogenic tumors (chondrosarcoma and osteosarcoma)and malignant fibrous histiocytoma, and some isoform of PP1 is believed to be involved in carcinogenesis. Sogawa et al. (1995) Cancer Letters 89:1-6;Sogawa et al., 1994, Res. Comm. in Mol. Pathol. and Pharmacol. 86:375-378; Yamada et al. (1994) Res. Comm. in Mol. Pathol. and Pharmacol. 86:125-128.

Certain abnormal proliferative or hyperproliferative conditions are believed to be associated with PP expression and are, therefore, believed to be responsive to inhibition of PP expression. Abnormally high levels of expression of the PP protein are implicated in carcinogenesis, i.e., the development of abnormal proliferative or hyperproliferative conditions. These abnormal conditions are also believed to be responsive to inhibition of PP expression. Examples of abnormal proliferative conditions are hyperproliferative disorders such as cancers, tumors and hyperplasias, including smooth muscle cell proliferation in the blood vessels, such as stenosis or restenosis following angioplasty. It is believed that elimination or reduction of PP expression may halt or reverse abnormal cell proliferation. This is believed to be true even when levels of PP expression are not abnormally high.

There is a great desire to provide compositions of matter which can modulate the expression of PPs. It is also desired to provide methods of detection of nucleic acids encoding PPs in cells, tissues and animals. It is also desired to provide methods of diagnosis and treatment of conditions associated with abnormal PP expression. In addition, kits and reagents for detection and study of

nucleic acids encoding PP are desired. "Abnormal" PP expression is defined herein as abnormally high levels of expression of the PP protein, expression of an abnormal or mutant PP protein, or any level of PP expression associated with an abnormal condition or state.

The present invention employs oligonucleotides targeted to nucleic acids encoding serine/threonine protein phosphatases. The relationship between an oligonucleotide and its complementary nucleic acid target to which it hybridizes is commonly referred to as "antisense". "Targeting" an oligonucleotide to a chosen nucleic acid target, in the context of this invention, is a multistep process. The process usually begins with identifying a nucleic acid sequence whose function is to be modulated. This may be, as examples, a cellular gene (or mRNA made from the gene) whose expression is associated with a particular disease state, or a foreign nucleic acid from an infectious agent. In the present invention, the target is a nucleic acid encoding a protein phosphatase; in other words, a protein phosphatase gene or mRNA expressed from a protein phosphatase gene. The targeting process also includes determination of a site or sites within the nucleic acid sequence for the oligonucleotide interaction to occur such that the desired effect- modulation of gene expression- will result. Once the target site or sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired modulation.

In the context of this invention "modulation" means either inhibition or stimulation. Inhibition of protein phosphatase gene expression is presently the preferred form of modulation. This modulation can be measured in ways which are routine in the art, for example by Northern blot

assay of mRNA expression or Western blot assay of protein expression as taught in the examples of the instant application. Effects on cell proliferation can also be measured, as taught in the examples of the instant application. "Hybridization", in the context of this invention, means hydrogen bonding, also known as Watson-Crick base pairing, between complementary bases, usually on opposite nucleic acid strands or two regions of a nucleic acid strand. Guanine and cytosine are examples of complementary bases which are known to form three hydrogen bonds between them. Adenine and thymine are examples of complementary bases which form two hydrogen bonds between them. "Specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment or, in the case of *in vitro* assays, under conditions in which the assays are conducted.

In preferred embodiments of this invention, oligonucleotides are provided which are targeted to mRNA encoding serine/threonine protein phosphatase 1γ1 (PP1γ1), serine/threonine protein phosphatase 4 (PP4) and serine/threonine protein phosphatase 5 (PP5). In

accordance with this invention, persons of ordinary skill in the art will understand that mRNA includes not only the coding region which carries the information to encode a protein using the three letter genetic code, including the translation start and stop codons, but also associated ribonucleotides which form a region known to such persons as the 5'-untranslated region, the 3'-untranslated region, the 5' cap region, intron regions and intron/exon or splice junction ribonucleotides. Thus, oligonucleotides may be formulated in accordance with this invention which are targeted wholly or in part to these associated ribonucleotides as well as to the coding ribonucleotides. In preferred embodiments, the oligonucleotide is targeted to a translation initiation site (AUG codon) or sequences in the coding region, 5' untranslated region or 3'-untranslated region of mRNA encoding human PP1γ1, PP4 and PP5. The functions of mRNA to be interfered with include all vital functions such as translocation of the RNA to the site for protein translation, actual translation of protein from the RNA, splicing or maturation of the RNA and possibly even independent catalytic activity which may be engaged in by the RNA. The overall effect of such interference with the RNA function is to cause interference with PP expression.

The present invention provides oligonucleotides for modulation of PP gene expression. Such oligonucleotides are targeted to nucleic acids encoding PPs. Oligonucleotides and methods for modulation of PP1γ1, PP4 and PP5 are provided; however, compositions and methods for modulating expression of other forms of serine/threonine protein phosphatases are also believed to have utility and are comprehended by this invention. As hereinbefore defined, "modulation" means either inhibition or

stimulation. Inhibition of PP gene expression is presently the preferred form of modulation.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages. The term "oligonucleotide" also includes oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake and increased stability in the presence of nucleases.

While the preferred form of antisense compound is a single-stranded antisense oligonucleotide, in many species the introduction of double-stranded structures, such as double-stranded RNA (dsRNA) molecules, has been shown to induce potent and specific antisense-mediated reduction of the function of a gene or its associated gene products. This phenomenon occurs in both plants and animals and is believed to have an evolutionary connection to viral defense and transposon silencing. Such double stranded RNA (and DNA) molecules are sithin the scope of the present invention.

The first evidence that dsRNA could lead to gene silencing in animals came in 1995 from work in the nematode, *Caenorhabditis elegans* (Guo and Kempheus, *Cell*, **1995**, 81, 611-620). Montgomery et al. have shown that the primary interference effects of dsRNA are posttranscriptional (Montgomery et al., *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 15502-15507). The posttranscriptional antisense mechanism defined in *Caenorhabditis elegans* resulting from exposure to double-stranded RNA (dsRNA) has since been designated RNA interference (RNAi). This term

has been generalized to mean antisense-mediated gene silencing involving the introduction of dsRNA leading to the sequence-specific reduction of endogenous targeted mRNA levels (Fire et al., *Nature*, **1998**, 391, 806-811). Recently, it has been shown that it is, in fact, the single-stranded RNA oligomers of antisense polarity of the dsRNAs which are the potent inducers of RNAi (Tijsterman et al., *Science*, **2002**, *295*, 694-697). The use of double stranded RNA (or DNA) molecules directed against PP5 (small interfering RNA, or siRNA) is also within the scope of the present invention.

Certain preferred oligonucleotides of this invention are chimeric oligonucleotides. "Chimeric oligonucleotides" or "chimeras", in the context of this invention, are oligonucleotides which contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at least one region of modified nucleotides that confers one or more beneficial properties (such as, for example, increased nuclease resistance, increased uptake into cells, increased binding affinity for the RNA target) and a region that is a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligos are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. In one preferred embodiment, a

chimeric oligonucleotide comprises at least one region modified to increase target binding affinity, and, usually, a region that acts as a substrate for RNAse H. Affinity of an oligonucleotide for its target (in this case a nucleic acid encoding a protein phosphatase) is routinely determined by measuring the Tm of an oligonucleotide/target pair, which is the temperature at which the oligonucleotide and target dissociate; dissociation is detected spectrophotometrically. The higher the Tm, the greater the affinity of the oligonucleotide for the target. In a more preferred embodiment, the region of the oligonucleotide which is modified to increase PP mRNA binding affinity comprises at least one nucleotide modified at the 2' position of the sugar, most preferably a 2'-O-alkyl, 2'-O-alkyl-O-alkyl or 2'-fluoro-modified nucleotide. Such modifications are routinely incorporated into oligonucleotides and these oligonucleotides have been shown to have a higher Tm (i.e., higher target binding affinity) than 2'-deoxyoligonucleotides against a given target.

The effect of such increased affinity is to greatly enhance antisense oligonucleotide inhibition of PP gene expression. RNAse H is a cellular endonuclease that cleaves the RNA strand of RNA:DNA duplexes; activation of this enzyme therefore results in cleavage of the RNA target, and thus can greatly enhance the efficiency of antisense inhibition. Cleavage of the RNA target can be routinely demonstrated by gel electrophoresis. In another preferred embodiment, the chimeric oligonucleotide is also modified to enhance nuclease resistance.

Cells contain a variety of exo- and endo-nucleases which can degrade nucleic acids. A number of nucleotide and nucleoside modifications have been shown to make the oligonucleotide into which they are incorporated more resistant to nuclease digestion than the native

oligodeoxynucleotide. Nuclease resistance is routinely measured by incubating oligonucleotides with cellular extracts or isolated nuclease solutions and measuring the extent of intact oligonucleotide remaining over time, usually by gel electrophoresis. Oligonucleotides which have been modified to enhance their nuclease resistance survive intact for a longer time than unmodified oligonucleotides. A variety of oligonucleotide modifications have been demonstrated to enhance or confer nuclease resistance. Oligonucleotides which contain at least one phosphorothioate modification are presently more preferred. In some cases, oligonucleotide modifications which enhance target binding affinity are also, independently, able to enhance nuclease resistance. A discussion of antisense oligonucleotides and some desirable modifications can be found in De Mesmaeker et al., 1995, Acc. Chem. Res. 28:366-374.

**Oligomer and Monomer Modifications**

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this linear polymeric compound can be further joined to form a circular compound, however, linear compounds are generally preferred. In addition, linear compounds may

have internal nucleobase complementarity and may therefore fold in a manner as to produce a fully or partially double-stranded compound. Within oligonucleotides, the phosphate groups are commonly referred to as forming the internucleoside linkage or in conjunction with the sugar ring the backbone of the oligonucleotide. The normal internucleoside linkage that makes up the backbone of RNA and DNA is a 3′ to 5′ phosphodiester linkage.

*Modified Internucleoside Linkages*

Specific examples of preferred antisense oligomeric compounds useful in this invention include oligonucleotides containing modified e.g. non-naturally occurring internucleoside linkages. As defined in this specification, oligonucleotides having modified internucleoside linkages include internucleoside linkages that retain a phosphorus atom and internucleoside linkages that do not have a phosphorus atom. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

In the *C. elegans* system, modification of the internucleotide linkage (phosphorothioate) did not significantly interfere with RNAi activity. Based on this observation, it is suggested that certain preferred oligomeric compounds of the invention can also have one or more modified internucleoside linkages. A preferred phosphorus containing modified internucleoside linkage is the phosphorothioate internucleoside linkage.

Preferred modified oligonucleotide backbones containing a phosphorus atom therein include, for example, phosphorothioates, chiral phosphorothioates, phosphoro-dithioates, phosphotriesters, aminoalkylphosphotriesters,

methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In more preferred embodiments of the invention, oligomeric compounds have one or more phosphorothioate and/or heteroatom internucleoside linkages, in particular -$CH_2$-NH-O-$CH_2$-, -$CH_2$-N($CH_3$)-O-$CH_2$- [known as a methylene (methylimino) or MMI backbone], -$CH_2$-O-N($CH_3$)-$CH_2$-, -$CH_2$-N($CH_3$)-N($CH_3$)-$CH_2$- and -O-N($CH_3$)-$CH_2$-$CH_2$- [wherein the native phosphodiester internucleotide linkage is represented as -

O-P(=O)(OH)-O-$CH_2$-]. The MMI type internucleoside linkages are disclosed in the above referenced U.S. patent 5,489,677. Preferred amide internucleoside linkages are disclosed in the above referenced U.S. patent 5,602,240.

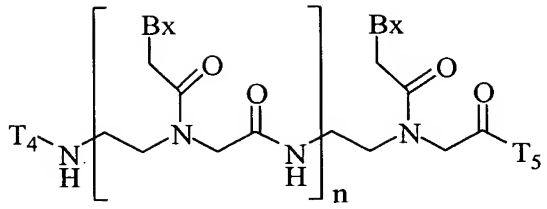
Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and $CH_2$ component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

*Oligomer Mimetics*

Another preferred group of oligomeric compounds amenable to the present invention includes oligonucleotide mimetics. The term mimetic as it is applied to oligonucleotides is intended to include oligomeric compounds wherein only the furanose ring or both the furanose ring and the internucleotide linkage are replaced with novel groups, replacement of only the furanose ring is also referred to in the art as being a sugar surrogate. The heterocyclic base moiety or a modified heterocyclic base moiety is maintained for hybridization with an appropriate target nucleic acid. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA oligomeric compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA oligomeric compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA oligomeric compounds can be found in Nielsen *et al.*, *Science*, **1991**, 254, 1497-1500.

PNA has been modified to incorporate numerous modifications since the basic PNA structure was first prepared. The basic structure is shown below:



wherein

Bx is a heterocyclic base moiety;

$T_4$ is hydrogen, an amino protecting group, $-C(O)R_5$, substituted or unsubstituted $C_1$-$C_{10}$ alkyl, substituted or unsubstituted $C_2$-$C_{10}$ alkenyl, substituted or unsubstituted $C_2$-$C_{10}$ alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional group, a reporter group, a conjugate group, a D or L α-amino acid linked via the α-carboxyl group or optionally through the ω-carboxyl group when the amino acid is aspartic acid or glutamic acid or a peptide derived from D, L or mixed D and L amino acids linked through a carboxyl group, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl;

$T_5$ is $-OH$, $-N(Z_1)Z_2$, $R_5$, D or L α-amino acid linked via the α-amino group or optionally through the ω-amino group when the amino acid is lysine or ornithine or a peptide derived from D, L or mixed D and L amino acids linked through an amino group, a chemical functional group, a reporter group or a conjugate group;

$Z_1$ is hydrogen, $C_1$-$C_6$ alkyl, or an amino protecting group;

$Z_2$ is hydrogen, $C_1$-$C_6$ alkyl, an amino protecting group, $-C(=O)-(CH_2)_n-J-Z_3$, a D or L α-amino acid linked via the α-carboxyl group or optionally through the ω-carboxyl group when the amino acid is aspartic acid or glutamic acid or a

peptide derived from D, L or mixed D and L amino acids linked through a carboxyl group;

$Z_3$ is hydrogen, an amino protecting group, $-C_1-C_6$ alkyl, $-C(=O)-CH_3$, benzyl, benzoyl, or $-(CH_2)_n-N(H)Z_1$;

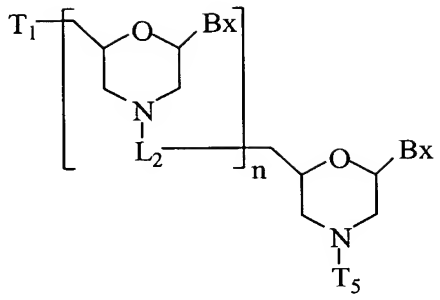
each J is O, S or NH;

$R_5$ is a carbonyl protecting group; and

n is from 2 to about 50.

Another class of oligonucleotide mimetic that has been studied is based on linked morpholino units (morpholino nucleic acid) having heterocyclic bases attached to the morpholino ring. A number of linking groups have been reported that link the morpholino monomeric units in a morpholino nucleic acid. A preferred class of linking groups have been selected to give a non-ionic oligomeric compound. The non-ionic morpholino-based oligomeric compounds are less likely to have undesired interactions with cellular proteins. Morpholino-based oligomeric compounds are non-ionic mimics of oligonucleotides which are less likely to form undesired interactions with cellular proteins (Dwaine A. Braasch and David R. Corey, *Biochemistry*, **2002**, *41(14)*, 4503-4510). Morpholino-based oligomeric compounds are disclosed in United States Patent 5,034,506, issued July 23, 1991. The morpholino class of oligomeric compounds have been prepared having a variety of different linking groups joining the monomeric subunits.

Morpholino nucleic acids have been prepared having a variety of different linking groups ($L_2$) joining the monomeric subunits. The basic formula is shown below:



wherein

$T_1$ is hydroxyl or a protected hydroxyl;

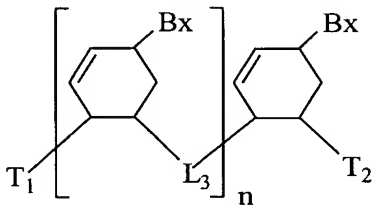
$T_5$ is hydrogen or a phosphate or phosphate derivative;

$L_2$ is a linking group; and

n is from 2 to about 50.

A further class of oligonucleotide mimetic is referred to as cyclohexenyl nucleic acids (CeNA). The furanose ring normally present in an DNA/RNA molecule is replaced with a cyclohenyl ring. CeNA DMT protected phosphoramidite monomers have been prepared and used for oligomeric compound synthesis following classical phosphoramidite chemistry. Fully modified CeNA oligomeric compounds and oligonucleotides having specific positions modified with CeNA have been prepared and studied (see Wang *et al., J. Am. Chem. Soc.*, **2000**, *122*, 8595-8602). In general the incorporation of CeNA monomers into a DNA chain increases its stability of a DNA/RNA hybrid. CeNA oligoadenylates formed complexes with RNA and DNA complements with similar stability to the native complexes. The study of incorporating CeNA structures into natural nucleic acid structures was shown by NMR and circular dichroism to proceed with easy conformational adaptation. Furthermore the incorporation of CeNA into a sequence targeting RNA was stable to serum and able to activate E. Coli RNase resulting in cleavage of the target RNA strand.

The general formula of CeNA is shown below:



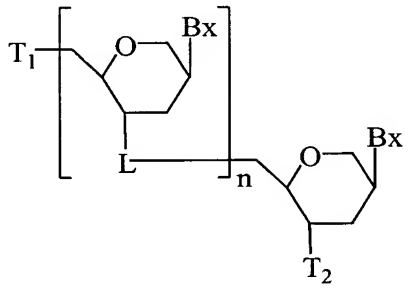

wherein

each Bx is a heterocyclic base moiety;

$T_1$ is hydroxyl or a protected hydroxyl; and

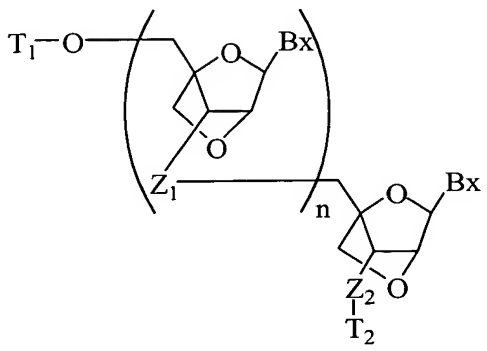
T2 is hydroxyl or a protected hydroxyl.

Another class of oligonucleotide mimetic (anhydrohexitol nucleic acid) can be prepared from one or more anhydrohexitol nucleosides (see, Wouters and Herdewijn, *Bioorg. Med. Chem. Lett.*, **1999**, *9*, 1563-1566) and would have the general formula:




A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 4' carbon atom of the sugar ring thereby forming a 2'-C,4'-C-oxymethylene linkage thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene $(-CH_2-)_n$ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2 (Singh et al., Chem. Commun., 1998, 4, 455-456). LNA and LNA analogs display very high duplex thermal stabilities with complementary DNA and RNA (Tm = +3 to +10 C), stability towards 3'-

exonucleolytic degradation and good solubility properties. The basic structure of LNA showing the bicyclic ring system is shown
below:




The conformations of LNAs determined by 2D NMR spectroscopy have shown that the locked orientation of the LNA nucleotides, both in single-stranded LNA and in duplexes, constrains the phosphate backbone in such a way as to introduce a higher population of the N-type conformation (Petersen et al., J. Mol. Recognit., 2000, 13, 44-53). These conformations are associated with improved stacking of the nucleobases (Wengel et al., Nucleosides Nucleotides, 1999, 18, 1365-1370).

LNA has been shown to form exceedingly stable LNA:LNA duplexes (Koshkin et al., J. Am. Chem. Soc., 1998, 120, 13252-13253). LNA:LNA hybridization was shown to be the most thermally stable nucleic acid type duplex system, and the RNA-mimicking character of LNA was established at the duplex level. Introduction of 3 LNA monomers (T or A) significantly increased melting points (Tm = +15/+11) toward DNA complements. The universality of LNA-mediated hybridization has been stressed by the formation of exceedingly stable LNA:LNA duplexes. The RNA-mimicking of LNA was reflected with regard to the N-type conformational

restriction of the monomers and to the secondary structure of the LNA:RNA duplex.

LNAs also form duplexes with complementary DNA, RNA or LNA with high thermal affinities. Circular dichroism (CD) spectra show that duplexes involving fully modified LNA (esp. LNA:RNA) structurally resemble an A-form RNA:RNA duplex. Nuclear magnetic resonance (NMR) examination of an LNA:DNA duplex confirmed the 3'-endo conformation of an LNA monomer. Recognition of double-stranded DNA has also been demonstrated suggesting strand invasion by LNA. Studies of mismatched sequences show that LNAs obey the Watson-Crick base pairing rules with generally improved selectivity compared to the corresponding unmodified reference strands.

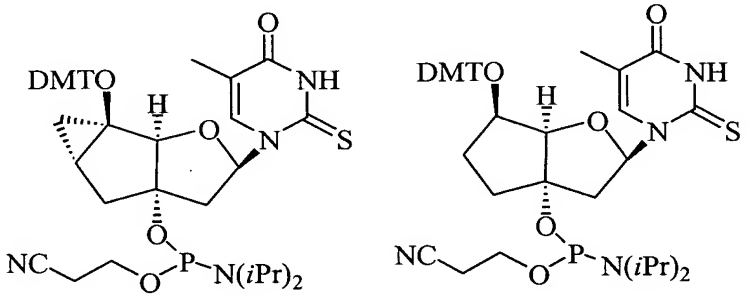
Novel types of LNA-oligomeric compounds, as well as the LNAs, are useful in a wide range of diagnostic and therapeutic applications. Among these are antisense applications, PCR applications, strand-displacement oligomers, substrates for nucleic acid polymerases and generally as nucleotide based drugs. Potent and nontoxic antisense oligonucleotides containing LNAs have been described (Wahlestedt et al., Proc. Natl. Acad. Sci. U. S. A., 2000, 97, 5633-5638.) The authors have demonstrated that LNAs confer several desired properties to antisense agents. LNA/DNA copolymers were not degraded readily in blood serum and cell extracts. LNA/DNA copolymers exhibited potent antisense activity in assay systems as disparate as G-protein-coupled receptor signaling in living rat brain and detection of reporter genes in Escherichia coli. Lipofectin-mediated efficient delivery of LNA into living human breast cancer cells has also been accomplished.

The synthesis and preparation of the LNA monomers adenine, cytosine, guanine, 5-methyl-cytosine, thymine and uracil, along with their oligomerization, and nucleic acid

recognition properties have been described (Koshkin et al., Tetrahedron, 1998, 54, 3607-3630). LNAs and preparation thereof are also described in WO 98/39352 and WO 99/14226.

The first analogs of LNA, phosphorothioate-LNA and 2'-thio-LNAs, have also been prepared (Kumar et al., Bioorg. Med. Chem. Lett., 1998, 8, 2219-2222). Preparation of locked nucleoside analogs containing oligodeoxyribonucleotide duplexes as substrates for nucleic acid polymerases has also been described (Wengel et al., PCT International Application WO 98-DK393 19980914). Furthermore, synthesis of 2'-amino-LNA, a novel conformationally restricted high-affinity oligonucleotide analog with a handle has been described in the art (Singh et al., J. Org. Chem., 1998, 63, 10035-10039). In addition, 2'-Amino- and 2'-methylamino-LNA's have been prepared and the thermal stability of their duplexes with complementary RNA and DNA strands has been previously reported.

Further oligonucleotide mimetics have been prepared to incude bicyclic and tricyclic nucleoside analogs having the formulas (amidite monomers shown):

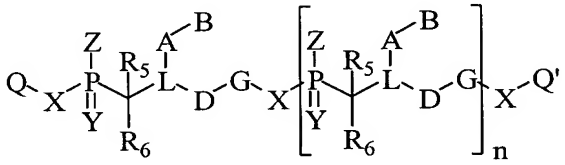



(see Steffens *et al., Helv. Chim. Acta*, **1997**, *80*, 2426-2439; Steffens *et al., J. Am. Chem. Soc.*, **1999**, *121*, 3249-3255; and Renneberg *et al., J. Am. Chem. Soc.*, **2002**, *124*, 5993-6002). These modified nucleoside analogs have been oligomerized using the phosphoramidite approach and the

resulting oligomeric compounds containing tricyclic nucleoside analogs have shown increased thermal stabilities (Tm's) when hybridized to DNA, RNA and itself. Oligomeric compounds containing bicyclic nucleoside analogs have shown thermal stabilities approaching that of DNA duplexes.

Another class of oligonucleotide mimetic is referred to as phosphonomonoester nucleic acids incorporate a phosphorus group in a backbone the backbone. This class of olignucleotide mimetic is reported to have useful physical and biological and pharmacological properties in the areas of inhibiting gene expression (antisense oligonucleotides, ribozymes, sense oligonucleotides and triplex-forming oligonucleotides), as probes for the detection of nucleic acids and as auxiliaries for use in molecular biology.

The general formula (for definitions of Markush variables see: United States Patents 5,874,553 and 6,127,346 herein incorporated by reference in their entirety) is shown below.



Another oligonucleotide mimetic has been reported wherein the furanosyl ring has been replaced by a cyclobutyl moiety.

*Modified sugars*

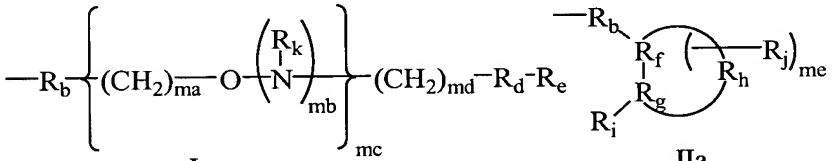
Oligomeric compounds of the invention may also contain one or more substituted sugar moieties. Preferred oligomeric compounds comprise a sugar substituent group selected from: OH; F; O-, S-, or N-alkyl; O-, S-, or N-

alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted $C_1$ to $C_{10}$ alkyl or $C_2$ to $C_{10}$ alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise a sugar substituent group selected from: $C_1$ to $C_{10}$ lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, $SCH_3$, OCN, Cl, Br, CN, $CF_3$, $OCF_3$, $SOCH_3$, $SO_2CH_3$, $ONO_2$, $NO_2$, $N_3$, $NH_2$, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al., Helv. Chim. Acta,* **1995**, *78*, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylamino-ethoxyethoxy (also known in the art as 2'-O-dimethyl-amino-ethoxy-ethyl or 2'-DMAEOE), i.e., 2'-O-$CH_2$-O-$CH_2$-$N(CH_3)_2$.

Other preferred sugar substituent groups include methoxy (-O-$CH_3$), aminopropoxy (-$OCH_2CH_2CH_2NH_2$), allyl (-$CH_2$-$CH=CH_2$), -O-allyl (-O-$CH_2$-$CH=CH_2$) and fluoro (F). 2'-Sugar substituent groups may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligomeric compound, particularly the 3' position of the sugar on the 3' terminal nucleoside or in

2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligomeric compounds may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Further representative sugar substituent groups include groups of formula $I_a$ or $II_a$:



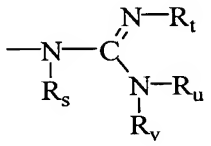
Ia IIa

wherein:

$R_b$ is O, S or NH;

$R_d$ is a single bond, O, S or C(=O);

$R_e$ is $C_1$-$C_{10}$ alkyl, $N(R_k)(R_m)$, $N(R_k)(R_n)$, $N=C(R_p)(R_q)$, $N=C(R_p)(R_r)$ or has formula $III_a$;



IIIa

$R_p$ and $R_q$ are each independently hydrogen or $C_1$-$C_{10}$ alkyl;

$R_r$ is $-R_x-R_y$;

each $R_s$, $R_t$, $R_u$ and $R_v$ is, independently, hydrogen, $C(O)R_w$, substituted or unsubstituted $C_1$-$C_{10}$ alkyl, substituted or unsubstituted $C_2$-$C_{10}$ alkenyl, substituted or unsubstituted $C_2$-$C_{10}$ alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional group or a conjugate group, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl;

or optionally, $R_u$ and $R_v$, together form a phthalimido moiety with the nitrogen atom to which they are attached;

each $R_w$ is, independently, substituted or unsubstituted $C_1$-$C_{10}$ alkyl, trifluoromethyl, cyanoethyloxy, methoxy, ethoxy, t-butoxy, allyloxy, 9-fluorenylmethoxy, 2-(trimethylsilyl)-ethoxy, 2,2,2-trichloroethoxy, benzyloxy, butyryl, iso-butyryl, phenyl or aryl;

$R_k$ is hydrogen, a nitrogen protecting group or $-R_x-R_y$;

$R_p$ is hydrogen, a nitrogen protecting group or $-R_x-R_y$;

$R_x$ is a bond or a linking moiety;

$R_y$ is a chemical functional group, a conjugate group or a solid support medium;

each $R_m$ and $R_n$ is, independently, H, a nitrogen protecting group, substituted or unsubstituted $C_1$-$C_{10}$ alkyl, substituted or unsubstituted $C_2$-$C_{10}$ alkenyl, substituted or unsubstituted $C_2$-$C_{10}$ alkynyl, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl, alkynyl; $NH_3^+$, $N(R_u)(R_v)$, guanidino and acyl where said acyl is an acid amide or an ester;

or $R_m$ and $R_n$, together, are a nitrogen protecting group, are joined in a ring structure that optionally

includes an additional heteroatom selected from N and O or are a chemical functional group;

$R_i$ is $OR_z$, $SR_z$, or $N(R_z)_2$;

each $R_z$ is, independently, H, $C_1$-$C_8$ alkyl, $C_1$-$C_8$ haloalkyl, $C(=NH)N(H)R_u$, $C(=O)N(H)R_u$ or $OC(=O)N(H)R_u$;

$R_f$, $R_g$ and $R_h$ comprise a ring system having from about 4 to about 7 carbon atoms or having from about 3 to about 6 carbon atoms and 1 or 2 heteroatoms wherein said heteroatoms are selected from oxygen, nitrogen and sulfur and wherein said ring system is aliphatic, unsaturated aliphatic, aromatic, or saturated or unsaturated heterocyclic;

$R_j$ is alkyl or haloalkyl having 1 to about 10 carbon atoms, alkenyl having 2 to about 10 carbon atoms, alkynyl having 2 to about 10 carbon atoms, aryl having 6 to about 14 carbon atoms, $N(R_k)(R_m)$ $OR_k$, halo, $SR_k$ or CN;

$m_a$ is 1 to about 10;

each mb is, independently, 0 or 1;

mc is 0 or an integer from 1 to 10;

md is an integer from 1 to 10;

me is from 0, 1 or 2; and

provided that when mc is 0, md is greater than 1.

Representative substituents groups of Formula I are disclosed in United States Patent Application Serial No. 09/130,973, filed August 7, 1998, entitled "Capped 2'-Oxyethoxy Oligonucleotides," hereby incorporated by reference in its entirety.

Representative cyclic substituent groups of Formula II are disclosed in United States Patent Application Serial No. 09/123,108, filed July 27, 1998, entitled "RNA Targeted 2'-Oligomeric compounds that are Conformationally Preorganized," hereby incorporated by reference in its entirety.

Particularly preferred sugar substituent groups include $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10.

Representative guanidino substituent groups that are shown in formula III and IV are disclosed in co-owned United States Patent Application 09/349,040, entitled "Functionalized Oligomers", filed July 7, 1999, hereby incorporated by reference in its entirety.

Representative acetamido substituent groups are disclosed in United States Patent 6,147,200 which is hereby incorporated by reference in its entirety.

Representative dimethylaminoethyloxyethyl substituent groups are disclosed in International Patent Application PCT/US99/17895, entitled "2'-O-Dimethylaminoethyloxyethyl-Oligomeric compounds", filed August 6, 1999, hereby incorporated by reference in its entirety.

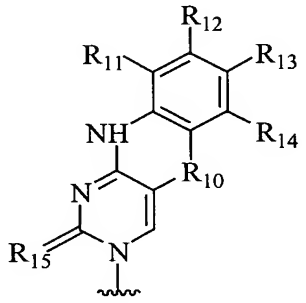
*Modified Nucleobases/Naturally occurring nucleobases*

Oligomeric compounds may also include nucleobase (often referred to in the art simply as "base" or "heterocyclic base moiety") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases also referred herein as heterocyclic base moieties include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-$CH_3$) uracil and cytosine and other alkynyl derivatives of

pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.

Heterocyclic base moieties may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, **1990**, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, **1991**, *30*, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B. , ed., CRC Press, **1993**. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyl-adenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, **1993**, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

In one aspect of the present invention oligomeric compounds are prepared having polycyclic heterocyclic compounds in place of one or more heterocyclic base moieties. A number of tricyclic heterocyclic comounds have been previously reported. These compounds are routinely used in antisense applications to increase the binding properties of the modified strand to a target strand. The most studied modifications are targeted to guanosines hence they have been termed G-clamps or cytidine analogs. Many of these polycyclic heterocyclic compounds have the general formula:




Representative cytosine analogs that make 3 hydrogen bonds with a guanosine in a second strand include 1,3-diazaphenoxazine-2-one ($R_{10}$ = O, $R_{11}$ - $R_{14}$= H) [Kurchavov, *et al., Nucleosides and Nucleotides*, 1997, 16, 1837-1846], 1,3-diazaphenothiazine-2-one ($R_{10}$= S, $R_{11}$ - $R_{14}$= H), [Lin, K.-Y.; Jones, R. J.; Matteucci, M. J. Am. Chem. Soc. 1995, 117, 3873-3874] and 6,7,8,9-tetrafluoro-1,3-diazaphenoxazine-2-one ($R_{10}$ = O, $R_{11}$ - $R_{14}$ = F) [Wang, J.; Lin, K.-Y., Matteucci, M. Tetrahedron Lett. 1998, 39, 8385-8388]. Incorporated into oligonucleotides these base modifications were shown to hybridize with complementary guanine and the latter was also shown to hybridize with adenine and to enhance helical thermal stability by

extended stacking interactions(also see U.S. Patent Application entitled "Modified Peptide Nucleic Acids" filed May 24, 2002, Serial number 10/155,920; and U.S. Patent Application entitled "Nuclease Resistant Chimeric Oligonucleotides" filed May 24, 2002, Serial number 10/013,295, both of which are commonly owned with this application and are herein incorporated by reference in their entirety).

Further helix-stabilizing properties have been observed when a cytosine analog/substitute has an aminoethoxy moiety attached to the rigid 1,3-diazaphenoxazine-2-one scaffold ($R_{10}$ = O, $R_{11}$ = -O-$(CH_2)_2$-$NH_2$, $R_{12-14}$=H ) [Lin, K.-Y.; Matteucci, M. J. Am. Chem. Soc. 1998, 120, 8531-8532]. Binding studies demonstrated that a single incorporation could enhance the binding affinity of a model oligonucleotide to its complementary target DNA or RNA with a $\Delta T_m$ of up to 18° relative to 5-methyl cytosine ($dC5^{me}$), which is the highest known affinity enhancement for a single modification, yet. On the other hand, the gain in helical stability does not compromise the specificity of the oligonucleotides. The $T_m$ data indicate an even greater discrimination between the perfect match and mismatched sequences compared to $dC5^{me}$. It was suggested that the tethered amino group serves as an additional hydrogen bond donor to interact with the Hoogsteen face, namely the O6, of a complementary guanine thereby forming 4 hydrogen bonds. This means that the increased affinity of G-clamp is mediated by the combination of extended base stacking and additional specific hydrogen bonding.

Further tricyclic heterocyclic compounds and methods of using them that are amenable to the present invention are disclosed in United States Patent Serial Number 6,028,183, which issued on May 22, 2000, and United States Patent Serial Number 6,007,992, which issued on December

28, 1999, the contents of both are commonly assigned with this application and are incorporated herein in their entirety.

The enhanced binding affinity of the phenoxazine derivatives together with their uncompromised sequence specificity makes them valuable nucleobase analogs for the development of more potent antisense-based drugs. In fact, promising data have been derived from in vitro experiments demonstrating that heptanucleotides containing phenoxazine substitutions are capable to activate RNaseH, enhance cellular uptake and exhibit an increased antisense activity [Lin, K-Y; Matteucci, M. J. Am. Chem. Soc. 1998, 120, 8531-8532]. The activity enhancement was even more pronounced in case of G-clamp, as a single substitution was shown to significantly improve the in vitro potency of a 20mer 2'-deoxyphosphorothioate oligonucleotides [Flanagan, W. M.; Wolf, J.J.; Olson, P.; Grant, D.; Lin, K.-Y.; Wagner, R. W.; Matteucci, M. Proc. Natl. Acad. Sci. USA, 1999, 96, 3513-3518]. Nevertheless, to optimize oligonucleotide design and to better understand the impact of these heterocyclic modifications on the biological activity, it is important to evaluate their effect on the nuclease stability of the oligomers.

Further modified polycyclic heterocyclic compounds useful as heterocyclcic bases are disclosed in but not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,434,257; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,646,269; 5,750,692; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, and Unites States Patent Application Serial number 09/996,292 filed November 28, 2001, certain of which are

commonly owned with the instant application, and each of which is herein incorporated by reference.

The oligonucleotides of the present invention also include variants in which a different base is present at one or more of the nucleotide positions in the oligonucleotide. For example, if the first nucleotide is an adenosine, variants may be produced which contain thymidine, guanosine or cytidine at this position. This may be done at any of the positions of the oligonucleotide. Thus, a 20-mer may comprise 60 variations (20 positions x 3 alternates at each position) in which the original nucleotide is substituted with any of the three alternate nucleotides. These oligonucleotides are then tested using the methods described herein to determine their ability to inhibit expression of HCV mRNA and/or HCV replication.

*Conjugates*

A further preferred substitution that can be appended to the oligomeric compounds of the invention involves the linkage of one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the resulting oligomeric compounds. In one embodiment such modified oligomeric compounds are prepared by covalently attaching conjugate groups to functional groups such as hydroxyl or amino groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of

this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992 the entire disclosure of which is incorporated herein by reference.

Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**, *86*, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Let.*, **1994**, *4*, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, **1992**, *660*, 306-309; Manoharan et al., *Bioorg. Med. Chem. Let.*, **1993**, *3*, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, **1992**, *20*, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, **1991**, *10*, 1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, *259*, 327-330; Svinarchuk et al., *Biochimie*, **1993**, *75*, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, *18*, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, **1995**, *14*, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, **1995**, *1264*, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, **1996**, *277*, 923-937.

The oligomeric compounds of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (*S*)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by reference in its entirety.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

*Chimeric oligomeric compounds*

It is not necessary for all positions in an oligomeric compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated

in a single oligomeric compound or even at a single monomeric subunit such as a nucleoside within a oligomeric compound. The present invention also includes oligomeric compounds which are chimeric oligomeric compounds. "Chimeric" oligomeric compounds or "chimeras," in the context of this invention, are oligomeric compounds that contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of a nucleic acid based oligomer.

Chimeric oligomeric compounds typically contain at least one region modified so as to confer increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligomeric compound may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligomeric compounds when chimeras are used, compared to for example phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric oligomeric compounds of the invention may be formed as composite structures of two or more oligonucleotides, oligonucleotide analogs, oligonucleosides and/or oligonucleotide mimetics as described above. Such oligomeric compounds have also been referred to in the art as hybrids hemimers, gapmers or inverted gapmers.

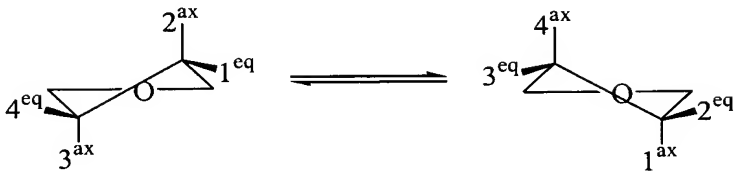
Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

*3'-endo modifications*

In one aspect of the present invention oligomeric compounds include nucleosides synthetically modified to induce a 3'-endo sugar conformation. A nucleoside can incorporate synthetic modifications of the heterocyclic base, the sugar moiety or both to induce a desired 3'-endo sugar conformation. These modified nucleosides are used to mimic RNA like nucleosides so that particular properties of an oligomeric compound can be enhanced while maintaining the desirable 3'-endo conformational geometry. There is an apparent preference for an RNA type duplex (A form helix, predominantly 3'-endo) as a requirement (e.g. trigger) of RNA interference which is supported in part by the fact that duplexes composed of 2'-deoxy-2'-F-nucleosides appears efficient in triggering RNAi response in the *C. elegans* system. Properties that are enhanced by using more stable 3'-endo nucleosides include but aren't limited to modulation of pharmacokinetic properties through modification of protein binding, protein off-rate, absorption and clearance; modulation of nuclease stability as well as chemical stability; modulation of the binding affinity and specificity of the oligomer (affinity and specificity for enzymes as well as for complementary sequences); and increasing efficacy of RNA cleavage. The present invention provides oligomeric triggers of RNAi

having one or more nucleosides modified in such a way as to favor a C3'-endo type conformation.

**Scheme 1**

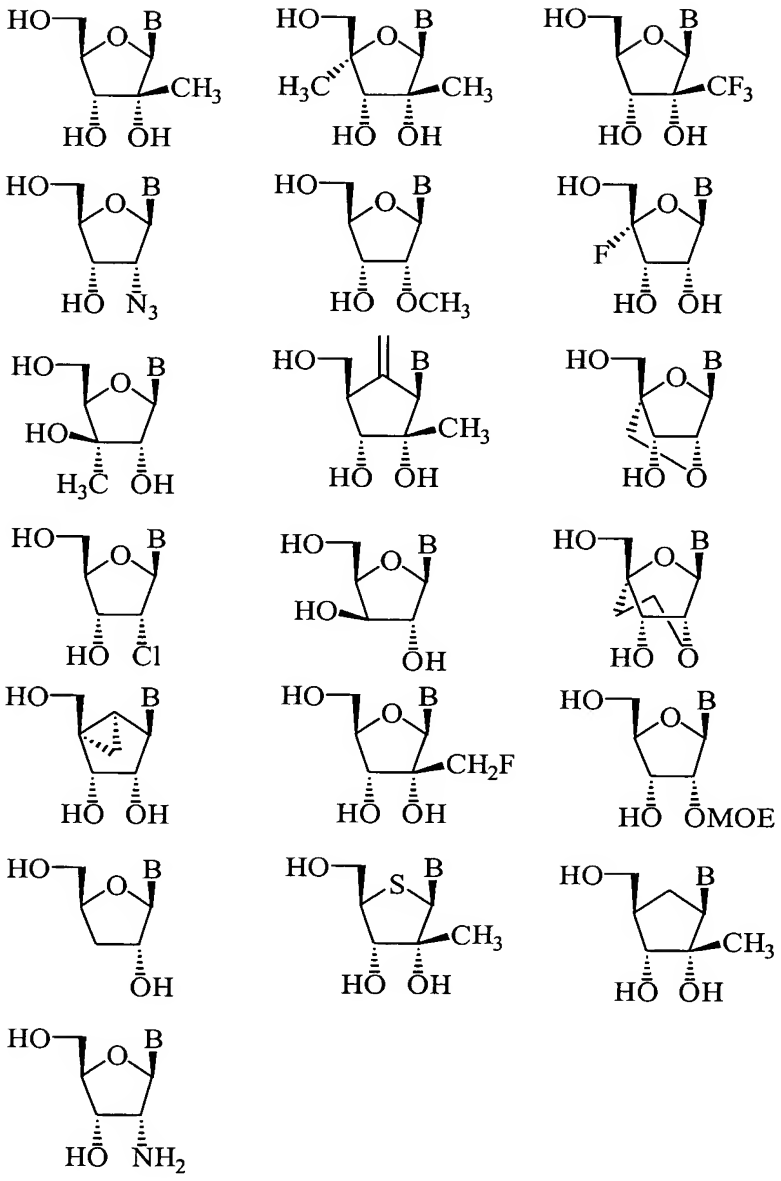



C2'-endo/Southern C3'-endo/Northern

Nucleoside conformation is influenced by various factors including substitution at the 2', 3' or 4'-positions of the pentofuranosyl sugar. Electronegative substituents generally prefer the axial positions, while sterically demanding substituents generally prefer the equatorial positions (Principles of Nucleic Acid Structure, Wolfgang Sanger, 1984, Springer-Verlag.) Modification of the 2' position to favor the 3'-endo conformation can be achieved while maintaining the 2'-OH as a recognition element, as illustrated in Figure 2, below (Gallo et al., Tetrahedron (2001), 57, 5707-5713. Harry-O'kuru et al., J. Org. Chem., (1997), 62(6), 1754-1759 and Tang et al., J. Org. Chem. (1999), 64, 747-754.) Alternatively, preference for the 3'-endo conformation can be achieved by deletion of the 2'-OH as exemplified by 2'deoxy-2'F-nucleosides (Kawasaki et al., J. Med. Chem. (1993), 36, 831-841), which adopts the 3'-endo conformation positioning the electronegative fluorine atom in the axial position. Other modifications of the ribose ring, for example substitution at the 4'-position to give 4'-F modified nucleosides (Guillerm et al., Bioorganic and Medicinal Chemistry Letters (1995), 5, 1455-1460 and Owen et al., J. Org. Chem. (1976), 41, 3010-3017), or for example modification to yield methanocarba nucleoside analogs (Jacobson et al., J.

Med. Chem. Lett. (2000), 43, 2196-2203 and Lee et al., Bioorganic and Medicinal Chemistry Letters (2001), 11, 1333-1337) also induce preference for the 3'-endo conformation. Along similar lines, oligomeric triggers of RNAi response might be composed of one or more nucleosides modified in such a way that conformation is locked into a C3'-endo type conformation, i.e. Locked Nucleic Acid (LNA, Singh et al, Chem. Commun. (1998), 4, 455-456), and ethylene bridged Nucleic Acids (ENA, Morita et al, Bioorganic & Medicinal Chemistry Letters (2002), 12, 73-76.) Examples of modified nucleosides amenable to the present invention are shown below. These examples are meant to be representative and not exhaustive.

5


HO B O CH3 HO OH
HO B O H3C CH3 HO OH
HO B O CF3 HO OH
HO B O HO N3
HO B O HO OCH3
HO B O F HO OH
HO B O HO H3C OH
HO B O CH3 HO OH
HO B O HO O
HO B O HO Cl
HO B O HO OH
HO B O HO O
HO B O HO OH
HO B O CH2F HO OH
HO B O HO OMOE
HO B O OH
HO S B CH3 HO OH
HO B CH3 HO OH
HO B O HO NH2

The preferred conformation of modified nucleosides and their oligomers can be estimated by various methods such as molecular dynamics calculations, nuclear magnetic resonance spectroscopy and CD measurements. Hence, modifications predicted to induce RNA like conformations, A-form duplex geometry in an oligomeric context, are selected for use in the modified oligoncleotides of the present invention. The synthesis of numerous of the modified nucleosides amenable to the present invention are known in the art (see for example, Chemistry of Nucleosides and Nucleotides Vol 1-3, ed. Leroy B. Townsend, 1988, Plenum press., and the examples section below.) Nucleosides known to be inhibitors/substrates for RNA dependent RNA polymerases (for example HCV NS5B

In one aspect, the present invention is directed to oligonucleotides that are prepared having enhanced properties compared to native RNA against nucleic acid targets. A target is identified and an oligonucleotide is selected having an effective length and sequence that is complementary to a portion of the target sequence. Each nucleoside of the selected sequence is scrutinized for possible enhancing modifications. A preferred modification would be the replacement of one or more RNA nucleosides with nucleosides that have the same 3'-endo conformational geometry. Such modifications can enhance chemical and nuclease stability relative to native RNA while at the same time being much cheaper and easier to synthesize and/or incorporate into an oligonulceotide. The selected sequence can be further divided into regions and the nucleosides of each region evaluated for enhancing modifications that can be the result of a chimeric configuration. Consideration is also given to the 5' and 3'-termini as there are often advantageous modifications that can be made to one or more of the terminal nucleosides. The oligomeric compounds of

the present invention include at least one 5'-modified phosphate group on a single strand or on at least one 5'-position of a double stranded sequence or sequences. Further modifications are also considered such as internucleoside linkages, conjugate groups, substitute sugars or bases, substitution of one or more nucleosides with nucleoside mimetics and any other modification that can enhance the selected sequence for its intended target.

The terms used to describe the conformational geometry of homoduplex nucleic acids are "A Form" for RNA and "B Form" for DNA. The respective conformational geometry for RNA and DNA duplexes was determined from X-ray diffraction analysis of nucleic acid fibers (Arnott and Hukins, *Biochem. Biophys. Res. Comm.*, **1970**, *47*, 1504.) In general, RNA:RNA duplexes are more stable and have higher melting temperatures (Tm's) than DNA:DNA duplexes (Sanger et al., Principles of Nucleic Acid Structure, 1984, Springer-Verlag; New York, NY.; Lesnik et al., Biochemistry, 1995, 34, 10807-10815; Conte et al., Nucleic Acids Res., 1997, 25, 2627-2634). The increased stability of RNA has been attributed to several structural features, most notably the improved base stacking interactions that result from an A-form geometry (Searle et al., Nucleic Acids Res., 1993, 21, 2051-2056). The presence of the 2' hydroxyl in RNA biases the sugar toward a C3' endo pucker, i.e., also designated as Northern pucker, which causes the duplex to favor the A-form geometry. In addition, the 2' hydroxyl groups of RNA can form a network of water mediated hydrogen bonds that help stabilize the RNA duplex (Egli et al., Biochemistry, 1996, 35, 8489-8494). On the other hand, deoxy nucleic acids prefer a C2' endo sugar pucker, i.e., also known as Southern pucker, which is thought to impart a less stable B-form geometry (Sanger, W. (1984) Principles of Nucleic Acid Structure, Springer-Verlag, New York, NY). As used

herein, B-form geometry is inclusive of both C2'-endo pucker and O4'-endo pucker. This is consistent with Berger, *et. al., Nucleic Acids Research*, **1998**, *26*, 2473-2480, who pointed out that in considering the furanose conformations which give rise to B-form duplexes consideration should also be given to a O4'-endo pucker contribution.

DNA:RNA hybrid duplexes, however, are usually less stable than pure RNA:RNA duplexes, and depending on their sequence may be either more or less stable than DNA:DNA duplexes (Searle *et al., Nucleic Acids Res.*, **1993**, *21*, 2051-2056). The structure of a hybrid duplex is intermediate between A- and B-form geometries, which may result in poor stacking interactions (Lane *et al., Eur. J. Biochem.*, **1993**, *215*, 297-306; Fedoroff *et al., J. Mol. Biol.*, **1993**, *233*, 509-523; Gonzalez *et al., Biochemistry*, **1995**, *34*, 4969-4982; Horton *et al., J. Mol. Biol.*, **1996**, *264*, 521-533). The stability of the duplex formed between a target RNA and a synthetic sequence is central to therapies such as but not limited to antisense and RNA interference as these mechanisms require the binding of a synthetic oligonucleotide strand to an RNA target strand. In the case of antisense, effective inhibition of the mRNA requires that the antisense DNA have a very high binding affinity with the mRNA. Otherwise the desired interaction between the synthetic oligonucleotide strand and target mRNA strand will occur infrequently, resulting in decreased efficacy.

One routinely used method of modifying the sugar puckering is the substitution of the sugar at the 2'-position with a substituent group that influences the sugar geometry. The influence on ring conformation is dependant on the nature of the substituent at the 2'-position. A number of different substituents have been studied to

determine their sugar puckering effect. For example, 2'-halogens have been studied showing that the 2'-fluoro derivative exhibits the largest population (65%) of the C3'-endo form, and the 2'-iodo exhibits the lowest population (7%). The populations of adenosine (2'-OH) versus deoxyadenosine (2'-H) are 36% and 19%, respectively. Furthermore, the effect of the 2'-fluoro group of adenosine dimers (2'-deoxy-2'-fluoroadenosine - 2'-deoxy-2'-fluoro-adenosine) is further correlated to the stabilization of the stacked conformation.

As expected, the relative duplex stability can be enhanced by replacement of 2'-OH groups with 2'-F groups thereby increasing the C3'-endo population. It is assumed that the highly polar nature of the 2'-F bond and the extreme preference for C3'-endo puckering may stabilize the stacked conformation in an A-form duplex. Data from UV hypochromicity, circular dichroism, and $^{1}H$ NMR also indicate that the degree of stacking decreases as the electronegativity of the halo substituent decreases. Furthermore, steric bulk at the 2'-position of the sugar moiety is better accommodated in an A-form duplex than a B-form duplex. Thus, a 2'-substituent on the 3'-terminus of a dinucleoside monophosphate is thought to exert a number of effects on the stacking conformation: steric repulsion, furanose puckering preference, electrostatic repulsion, hydrophobic attraction, and hydrogen bonding capabilities. These substituent effects are thought to be determined by the molecular size, electronegativity, and hydrophobicity of the substituent. Melting temperatures of complementary strands is also increased with the 2'-substituted adenosine diphosphates. It is not clear whether the 3'-endo preference of the conformation or the presence of the substituent is responsible for the increased binding.

However, greater overlap of adjacent bases (stacking) can be achieved with the 3'-endo conformation.

One synthetic 2'-modification that imparts increased nuclease resistance and a very high binding affinity to nucleotides is the 2-methoxyethoxy (2'-MOE, 2'-$OCH_2CH_2OCH_3$) side chain (Baker *et al.*, *J. Biol. Chem.*, **1997**, *272*, 11944-12000). One of the immediate advantages of the 2'-MOE substitution is the improvement in binding affinity, which is greater than many similar 2' modifications such as *O*-methyl, *O*-propyl, and *O*-aminopropyl. Oligonucleotides having the 2'-*O*-methoxyethyl substituent also have been shown to be antisense inhibitors of gene expression with promising features for *in vivo* use (Martin, P., *Helv. Chim. Acta*, **1995**, *78*, 486-504; Altmann *et al.*, *Chimia*, **1996**, *50*, 168-176; Altmann *et al.*, *Biochem. Soc. Trans.*, **1996**, *24*, 630-637; and Altmann *et al.*, *Nucleosides Nucleotides*, 1997, ***16***, 917-926). Relative to DNA, the oligonucleotides having the 2'-MOE modification displayed improved RNA affinity and higher nuclease resistance. Chimeric oligonucleotides having 2'-MOE substituents in the wing nucleosides and an internal region of deoxy-phosphorothioate nucleotides (also termed a gapped oligonucleotide or gapmer) have shown effective reduction in the growth of tumors in animal models at low doses. 2'-MOE substituted oligonucleotides have also shown outstanding promise as antisense agents in several disease states. One such MOE substituted oligonucleotide is presently being investigated in clinical trials for the treatment of CMV retinitis.

**Chemistries Defined**

Unless otherwise defined herein, alkyl means $C_1$-$C_{12}$, preferably $C_1$-$C_8$, and more preferably $C_1$-$C_6$, straight or (where possible) branched chain aliphatic hydrocarbyl.

Unless otherwise defined herein, heteroalkyl means $C_1$-$C_{12}$, preferably $C_1$-$C_8$, and more preferably $C_1$-$C_6$, straight or (where possible) branched chain aliphatic hydrocarbyl containing at least one, and preferably about 1 to about 3, hetero atoms in the chain, including the terminal portion of the chain. Preferred heteroatoms include N, O and S.

Unless otherwise defined herein, cycloalkyl means $C_3$-$C_{12}$, preferably $C_3$-$C_8$, and more preferably $C_3$-$C_6$, aliphatic hydrocarbyl ring.

Unless otherwise defined herein, alkenyl means $C_2$-$C_{12}$, preferably $C_2$-$C_8$, and more preferably $C_2$-$C_6$ alkenyl, which may be straight or (where possible) branched hydrocarbyl moiety, which contains at least one carbon-carbon double bond.

Unless otherwise defined herein, alkynyl means $C_2$-$C_{12}$, preferably $C_2$-$C_8$, and more preferably $C_2$-$C_6$ alkynyl, which may be straight or (where possible) branched hydrocarbyl moiety, which contains at least one carbon-carbon triple bond.

Unless otherwise defined herein, heterocycloalkyl means a ring moiety containing at least three ring members, at least one of which is carbon, and of which 1, 2 or three ring members are other than carbon. Preferably the number of carbon atoms varies from 1 to about 12, preferably 1 to about 6, and the total number of ring members varies from three to about 15, preferably from about 3 to about 8. Preferred ring heteroatoms are N, O and S. Preferred heterocycloalkyl groups include morpholino, thiomorpholino, piperidinyl, piperazinyl, homopiperidinyl, homopiperazinyl, homomorpholino, homothiomorpholino, pyrrolodinyl, tetrahydrooxazolyl, tetrahydroimidazolyl, tetrahydrothiazolyl, tetrahydroisoxazolyl, tetrahydropyrrazolyl, furanyl, pyranyl, and tetrahydroisothiazolyl.

Unless otherwise defined herein, aryl means any hydrocarbon ring structure containing at least one aryl ring. Preferred aryl rings have about 6 to about 20 ring carbons. Especially preferred aryl rings include phenyl, napthyl, anthracenyl, and phenanthrenyl.

Unless otherwise defined herein, hetaryl means a ring moiety containing at least one fully unsaturated ring, the ring consisting of carbon and non-carbon atoms. Preferably the ring system contains about 1 to about 4 rings. Preferably the number of carbon atoms varies from 1 to about 12, preferably 1 to about 6, and the total number of ring members varies from three to about 15, preferably from about 3 to about 8. Preferred ring heteroatoms are N, O and S. Preferred hetaryl moieties include pyrazolyl, thiophenyl, pyridyl, imidazolyl, tetrazolyl, pyridyl, pyrimidinyl, purinyl, quinazolinyl, quinoxalinyl, benzimidazolyl, benzothiophenyl, etc.

Unless otherwise defined herein, where a moiety is defined as a compound moiety, such as hetarylalkyl (hetaryl and alkyl), aralkyl (aryl and alkyl), etc., each of the sub-moieties is as defined herein.

Unless otherwise defined herein, an electron withdrawing group is a group, such as the cyano or isocyanato group that draws electronic charge away from the carbon to which it is attached. Other electron withdrawing groups of note include those whose electronegativities exceed that of carbon, for example halogen, nitro, or phenyl substituted in the ortho- or para-position with one or more cyano, isothiocyanato, nitro or halo groups.

Unless otherwise defined herein, the terms halogen and halo have their ordinary meanings. Preferred halo (halogen) substituents are Cl, Br, and I.

The aforementioned optional substituents are, unless otherwise herein defined, suitable substituents depending

upon desired properties. Included are halogens (Cl, Br, I), alkyl, alkenyl, and alkynyl moieties, $NO_2$, $NH_3$ (substituted and unsubstituted), acid moieties (e.g. $-CO_2H$, $-OSO_3H_2$, etc.), heterocycloalkyl moieties, hetaryl moieties, aryl moieties, etc. In all the preceding formulae, the squiggle (~) indicates a bond to an oxygen or sulfur of the 5'-phosphate.

Phosphate protecting groups include those described in US Patents No. US 5,760,209, US 5,614,621, US 6,051,699, US 6,020,475, US 6,326,478, US 6,169,177, US 6,121,437, US 6,465,628 each of which is expressly incorporated herein by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the *in vivo* synthesis of antisense molecules.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting

formulations include, but are not limited to, U.S. Patents 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, **1977**, *66*, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid,

methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfoc acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid,

tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an anima

Pharmaceutically Acceptable Salts: The term pharmaceutically acceptable salts refers to physiologically and pharmaceutically acceptable salts of the oligonucleotides of the invention: *i.e.*, salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge *et al.*, "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, *66:*1). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used

herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth,

ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, *etc.*; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The oligonucleotides in accordance with this invention preferably are from about 8 to about 50 nucleotides in length. In the context of this invention it is understood that this encompasses non-naturally occurring oligomers as hereinbefore described, having 8 to 50 monomers.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the talents of the routineer. It is also well known to use similar techniques to prepare other oligonucleotides such as the

phosphorothioates and alkylated derivatives. It is also well known to use similar techniques and commercially available modified amidites and controlled-pore glass (CPG) products such as biotin, fluorescein, acridine or psoralen-modified amidites and/or CPG (available from Glen Research, Sterling VA) to synthesize fluorescently labeled, biotinylated or other modified oligonucleotides such as cholesterol-modified oligonucleotides.

It has now been found that certain oligonucleotides targeted to portions of PP1γ1, PP4 and PP5 mRNA are useful for inhibiting PP expression. Inhibition of PP expression using antisense oligonucleotides is believed to be useful for interfering with cell hyperproliferation. In the methods of the invention, tissues or cells are contacted with oligonucleotides. In the context of this invention, to "contact" tissues or cells with an oligonucleotide or oligonucleotides means to add the oligonucleotide(s), usually in a liquid carrier, to a cell suspension or tissue sample, either *in vitro* or *ex vivo*, or to administer the oligonucleotide(s) to cells or tissues within an animal.

For therapeutics, methods of inhibiting hyperproliferation of cells and methods of preventing and treating abnormal proliferative conditions are provided. The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill in the art. In general, for therapeutics, a patient suspected of needing such therapy is given an oligonucleotide in accordance with the invention, commonly in a pharmaceutically acceptable carrier, in amounts and for periods which will vary depending upon the nature of the particular disease, its severity and the patient's overall condition. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is

desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral or parenteral. Parenteral administration includes intravenous drip or infusion, subcutaneous, intraperitoneal or intramuscular injection, pulmonary administration, e.g., by inhalation or insufflation, or intrathecal or intraventricular administration. For oral administration, it has been found that oligonucleotides with at least one 2'-substituted ribonucleotide are particularly useful because of their absortion and distribution characteristics. U.S. Patent 5,591,721 (Agrawal et al.). Oligonucleotides with at least one 2'-methoxyethyl modification are believed to be particularly useful for oral administration.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

In addition to such pharmaceutical carriers, cationic lipids may be included in the formulation to facilitate oligonucleotide uptake. One such composition shown to facilitate uptake is Lipofectin (BRL, Bethesda MD).

Dosing is dependent on severity and responsiveness of the condition to be treated, with course of treatment lasting from several days to several months or until a cure is effected or a diminution of disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be calculated based on $IC_{50}$'s or $EC_{50}$'s in *in vitro* and *in vivo* animal studies. For example, given the molecular weight of compound (derived from oligonucleotide sequence and chemical structure) and an effective dose such as an $IC_{50}$, for example (derived experimentally), a dose in mg/kg is routinely calculated.

The present invention is also suitable for diagnosing abnormal proliferative states in tissue or other samples from patients suspected of having a hyperproliferative disease such as cancer. The ability of the oligonucleotides of the present invention to inhibit cell proliferation may be employed to diagnose such states. A number of assays may be formulated employing the present invention, which assays will commonly comprise contacting a tissue sample with an oligonucleotide of the invention under conditions selected to permit detection and, usually, quantitation of such inhibition. Similarly, the present invention can be used to distinguish PP-associated, or, particularly, PP1γ1, PP4 or PP5-associated tumors from tumors having other etiologies, in order that an efficacious treatment regime can be designed.

The oligonucleotides of this invention may also be used for research purposes. Thus, the specific hybridization exhibited by the oligonucleotides may be used for assays, purifications, cellular product preparations

and in other methodologies which may be appreciated by persons of ordinary skill in the art.

The oligonucleotides of the invention are also useful for detection and diagnosis of PP expression. For example, radiolabeled oligonucleotides can be prepared by $^{32}P$ labeling at the 5' end with polynucleotide kinase. Sambrook et al., *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989, Volume 2, p. 10.59. Radiolabeled oligonucleotides are then contacted with tissue or cell samples suspected of PP expression and the sample is washed to remove unbound oligonucleotide. Radioactivity remaining in the sample indicates bound oligonucleotide (which in turn indicates the presence of PP) and can be quantitated using a scintillation counter or other routine means. Radiolabeled oligo can also be used to perform autoradiography of tissues to determine the localization, distribution and quantitation of PP expression for research, diagnostic or therapeutic purposes. In such studies, tissue sections are treated with radiolabeled oligonucleotide and washed as described above, then exposed to photographic emulsion according to routine autoradiography procedures. The emulsion, when developed, yields an image of silver grains over the regions expressing PP. Quantitation of the silver grains permits PP expression to be detected.

Analogous assays for fluorescent detection of PP expression can be developed using oligonucleotides of the invention which are conjugated with fluorescein or other fluorescent tag instead of radiolabeling. Such conjugations are routinely accomplished during solid phase synthesis using fluorescently labeled amidites or CPG (e.g., fluorescein-labeled amidites and CPG available from Glen Research, Sterling VA. See 1993 Catalog of Products for DNA Research, Glen Research, Sterling VA, p. 21).

Each of these assay formats is known in the art. One of skill could easily adapt these known assays for detection of PP expression in accordance with the teachings of the invention providing a novel and useful means to detect PP expression.

**Oligonucleotide inhibition of PP5 expression**

The oligonucleotides shown in Table 1 were designed using the Genbank sequences HSSERTHRP (Genbank accession number X92121, SEQ ID NO: 38), HSRNAPPP5 (Genbank accession number X89416, SEQ ID NO: 39) and PPP5C (Genbank accession number U25174, SEQ ID NO: 40), synthesized and tested for inhibition of PP5 mRNA expression in A549 cells using a Northern blot assay. All oligonucleotides shown in Table 1 are chimeric oligonucleotides with central 2'-deoxy "gaps," which have phosphorothioate backbones, flanked on both sides by 2'-methoxyethoxy (2'-MOE) "wings" (shown in **bold**), which have phosphodiester backbones. All cytosines in the 2'-MOE wings are 5-methylcytosines.

Table 1

Human PP5 Antisense Oligonucleotides

| Isis # | Sequence (5' 6 3') | Target source and site (Genbank #; nucleotide #s) | Target region | Percent Inhibition | SEQ ID NO: |
|---|---|---|---|---|---|
| 14493 | **TCGCCC**TCCGCCAT**CGCCAT** | x92121; nt 70-89; AUG | AUG | **84%** | 1 |
| 14494 | **TTCAGA**GCTCCATC**AGCCGG** | x92121; nt 127-146; | coding | **52** | 2 |
| 14495 | **GTAGGC**CAGGCTGC**GGTTGC** | u25174; nt 175-194; | coding | **66** | 3 |
| 14496 | **CCGCTG**TACTCATC**CTCAAT** | u25174; nt 492-511; | coding | **54** | 4 |
| 14497 | **TCCCCA**CATACTGT**AATCTT** | u25174; nt 684-703; | coding | 11 | 5 |

| | | | | | |
|---|---|---|---|---|---|
| 14498 | **GTACT**TGGCCTTCA**CCTCAC** | x89416; nt 933-952; | coding | **80** | 6 |
| 14499 | **CCAGG**TTGTTCTCTT**CCAAG** | x89416; nt 1225-1244; | coding | **62** | 7 |
| 14500 | **AGAGCC**CTGGAGGT**GGATGT** | x89416; nt 1365-1384; | coding | 41 | 8 |
| 14501 | **CGCCCC**GCCCGTCA**CCTCAC** | x89416; nt 1480-1499; | Stop codon | 42 | 9 |
| 14502 | **CCTACC**CCCTCTGC**AAACCT** | x89416; nt 1625-1644; | 3' UTR | 40 | 10 |
| 14503 | **GCCCCA**GCTGCTCC**ACCTCC** | x89416; nt 1694-1713; | 3' UTR | 27 | 11 |
| 14504 | **GGGCCC**TATTGCTT**GAGTGG** | x89416; nt 1810-1829; | 3' UTR | **92** | 12 |
| 14505 | **CCCAGC**CTAGCCCC**ACCATG** | x89416; nt 1899-1918; | 3' UTR | 23 | 13 |

In the initial screen, A549 cells were treated with oligonucleotides at a concentration of 300 nM oligonucleotide for four hours in the presence of 20 mg/ml lipofectin. Results were normalized and expressed as a percent of control. The effect of each oligonucleotide on levels of PP5 mRNA, expressed as approximate percent inhibition compared to control, is shown in Table 1. In this initial screen, oligonucleotides giving a reduction of PP5 mRNA of approximately 50% or greater were considered active. According to this criterion, oligonucleotides 14493, 14494, 14495, 14496, 14498, 14499 and 14504 were found to be active. These sequences (SEQ ID NO: 1, 2, 3, 4, 6, 7 and 12, respectively, SEQ ID Nos shown in **bold** in Table 1) are therefore preferred. Of these, oligonucleotides 14493, 14498 and 14504 (SEQ ID NO: 1, 6 and 12, respectively) showed at least 70% inhibition of PP5 mRNA in this assay and are highly active.

**Additional oligonucleotides targeted to PP5:**

Additional oligonucleotides targeted to PP5 and having SEQ ID NO: 12 were synthesized. These are chimeric oligonucleotides having slightly wider deoxy gaps (and

shorter 2'-MOE wings, shown in **bold**) than ISIS 14504. These oligonucleotides are shown in Table 2, along with ISIS 15521, a mismatch control:

Table 2

| Isis # | Sequence | Backbone | SEQ ID NO: |
|---|---|---|---|
| 15523 | **GGGCC**CTATTGCTTG**AGTGG** | P=S | 12 |
| 15534 | **GGGCC**CTATTGCTTG**AGTGG** | P=O/P=S | 12 |
| 15521 | **GTGCG**ATCGTTGCGG**TTAGC** | P=O/P=S | 14 |

These oligonucleotides differ in their backbone composition; ISIS 15523 is uniformly phosphorothioate (P=S) and ISIS 15534 is a mixed backbone compound with a phosphodiester backbone (P=O) in the wings and phosphorothioate (P=S) in the deoxy gap. ISIS 15521, the mismatch control, is also a mixed backbone compound with phosphodiester wings and a phosphorothiate gap.

These oligonucleotides were tested for their ability to reduce PP5 mRNA levels in A549 cells, using oligonucleotide doses of 25 to 500 nM. ISIS 15523 demonstrated an $IC_{50}$ of approximately 100 nM, and ISIS 15534 demonstrated an $IC_{50}$ of approximately 135 nM. The mismatch control, ISIS 15521, did not inhibit PP5 mRNA levels by more than 20% at any of the doses tested.

**Effect of antisense inhibition of PP5 expression on cell proliferation:**

A549 cells were treated with ISIS 15534 or its scrambled control, ISIS 15521 at a concentration of 300 nM. Each day for the next 5 days, viable cells were counted. The scrambled control oligonucleotide, ISIS 11521, was approximately equivalent to untreated control on all 5 days. In contrast, the cells treated with ISIS 15534 showed

markedly decreased proliferation compared to untreated cells. On days 2, 3, 4 and 5, ISIS 15534-treated cells showed a decrease in proliferation of 55%, 75%, 89% and 55%, respectively, compared to control.

**Effect of antisense inhibition of PP5 expression on DNA replication:**

A549 cells were treated with ISIS 15534 or its scrambled control, ISIS 15521 at a concentration of 300 nM. Cells were pulse-labeled with [$^3$H]-thymidine for 5 hours at intervals over the next five days. Cells were lysed and [$^3$H]-thymidine incorporation (indicative of DNA synthesis) was determined by liquid scintillation counting. The scrambled control oligonucleotide, ISIS 11521, was approximately equivalent to untreated control on all 5 days. In contrast, the cells treated with ISIS 15534 showed markedly decreased thymidine incorporation compared to untreated cells. On days 2, 3, 4 and 5, ISIS 15534-treated cells showed a decrease in [$^3$H]-thymidine incorporation of approximately 85%, 88% and 62%, respectively, compared to control. By day 5 [$^3$H]-thymidine incorporation was approximately equivalent in treated and untreated cells.

**Additional oligonucleotides targeted to PP5:**

An additional oligonucleotides targeted to PP5 and having SEQ ID NO: 1 was synthesized. This compound, ISIS 15516 has a phosphorothioate backbone and is a chimeric oligonucleotide having a slightly wider deoxy gap (and shorter 2'-MOE wings, shown in **bold**) than ISIS 14493. These oligonucleotides are shown in Table 3, along with ISIS 15517, a mismatch control with a mixed backbone (P=S in the gap, P=O in the wings):

Table 3

| Isis # | Sequence | Backbone | SEQ ID NO: |
|---|---|---|---|
| 14493 | **TCGCCC**TCCGCCAT**CGCCAT** | P=O/P=S | 1 |
| 15516 | **TCGCC**CTCCGCCATC**GCCAT** | P=S | 1 |
| 15517 | **GCTCT**ACTCCGCCCC**ATGCC** | P=O/P=S | 15 |

ISIS 15516 and 15517 oligonucleotides were tested for their ability to reduce PP5 mRNA levels in RINm5f cells. Dose response curves were generated for oligonucleotide doses of 25 to 500 nM. ISIS 15516 demonstrated an $IC_{50}$ of approximately 135 nM. The scrambled control, ISIS 15517, gave less than 10% reduction of PP5 mRNA levels at any dose tested.

RINm5f cells are an insulin-secreting insulinoma rat cell line. Previous studies indicate that there is a correlation between cell growth, insulin secretion, calcium channel activity and phosphatases in RINm5f cells. In both humans and rat, calcium channels are phosphorylated, and phosphorylation is believed to keep the channel closed (i.e., phosphorylation causes a decrease in the frequency and/or duration of channel opening). There is substantial evidence that calcium channels are involved in the regulation of insulin secretion. Calcium channel blockers such as nifedipine and verapamil are used in the treatment of cardiac disorders, such as angina, congestive heart failure and certain arrhythmias, as well as hypertension. Thus agents that affect calcium channels, particularly calcium channel blockers, are believed to be therapeutically useful.

The antisense oligonucleotide ISIS 15516 (SEQ ID NO: 1), targeted to the AUG region of human PP5, was tested in RINm5f cells for its effect on calcium channels, using standard patch-clamp techniques. Because the human and rat

PP5 mRNA sequences are identical in the target region of this oligonucleotide, ISIS 15516 is perfectly complementary to this portion of the rat PP5 sequence. Treatment of RINm5f cells with a 300 nM concentration of ISIS 15516 indicated that this compound decreases calcium currents in these cells. The mismatch control oligonucleotide, ISIS 15517, did not show this effect. Since inhibition of PP5 expression by ISIS 15516 is now shown to decrease calcium current density, it is believed that this compound and other inhibitors of PP5 may be useful as calcium channel blockers, for example in treatment of cardiac conditions.

**Oligonucleotide inhibition of PP4 expression**

The oligonucleotides shown in Table 4, targeted to human PP4 (also known as protein phosphatase X) were designed using the Genbank sequence HSPPX (Genbank accession number X70218, SEQ ID NO: 41), synthesized and tested for inhibition of PP4 mRNA expression in A549 cells using a Northern blot assay. All oligonucleotides shown in Table 4 are chimeric oligonucleotides with central 2'-deoxy "gaps," which have phosphorothioate backbones, flanked on both sides by 2'-methoxyethoxy (2'-MOE) "wings" (shown in bold), which have phosphodiester backbones. All cytosines in the 2'-MOE wings are 5-methylcytosines.

Table 4

Human PP4 Antisense Oligonucleotides

| Isis # | Sequence (5' 6 3') | Target source and site (Genbank #; nucleotide #s) | Target region | Percent Inhibition | SEQ ID NO: |
|---|---|---|---|---|---|
| 14375 | **CCATG**GCCCACCCCC**GGCGC** | X70218;123-142 | AUG | 85.6% | 16 |
| 14376 | **TGATC**TCCGCCATGG**CCCAC** | X70218; 132-151 | AUG | 81.4 | 17 |

| | | | | | |
|---|---|---|---|---|---|
| 14377 | **CGGTC**CACAAAGTCC**CCCAT** | X70218;376-395 | coding | 7.9 | 18 |
| 14378 | **GAGGC**CCCCGTGCAC**GCAGA** | X70218;620-639 | coding | 61.9 | **19** |
| 14379 | **ACGTC**ACTGCCAAAT**AGGTA** | X70218;781-800 | coding | 78.3 | **20** |
| 14380 | **TGCCA**CATTCCCACA**GCGGT** | X70218;929-948 | coding | 59.9 | **21** |
| 14381 | **GGGAG**CAGCCTCAAA**GATGA** | X70218;989-1008 | coding | 48.5 | 22 |
| 14383 | **GATGG**CAGAGTCACA**GTGGT** | X70218;1105-1124 | 3' UTR | 63.1 | **23** |
| 14385 | **GGGAC**AGCAGAGCCA**GGACA** | X70218;1150-1169 | 3' UTR | 57.8 | **24** |
| 14387 | **AACTT**CATGGTTCAA**GTGGG** | X70218;1247-1266 | 3' UTR | 78.1 | **25** |

In this initial screen, oligonucleotides giving a reduction of PP4 mRNA of approximately 50% or greater were considered active. According to this criterion, oligonucleotides 14375, 14376, 14378, 14379, 14380, 14383, 14385 and 14387 were found to be active. These sequences (SEQ ID NO: 16, 17, 19, 20, 21, 23, 24 and 25, respectively, SEQ ID NOs shown in **bold** in Table 4) are therefore preferred. Of these, oligonucleotides 14375, 14376, 14379 and 14387 (SEQ ID NO: 16, 17, 20, and 25, respectively) showed at least 70% inhibition of PP1γ1 mRNA in this assay and are highly active.

**Oligonucleotide inhibition of PP1 expression**

The oligonucleotides shown in Table 5, targeted to human PP1γ1 were designed using the Genbank sequence HSPPICC (Genbank accession number X74008, SEQ ID NO: 42), synthesized and tested for inhibition of PP1γ1 mRNA expression in A549 cells using a Northern blot assay. All oligonucleotides shown in Table 5 are chimeric oligonucleotides with central 2'-deoxy "gaps," which have phosphorothioate backbones, flanked on both sides by 2'-methoxyethoxy (2'-MOE) "wings" (shown in **bold**), which have phosphodiester backbones. All cytosines in the 2'-MOE wings

are 5-methylcytosines. Oligonucleotides were tested at a concentration of 300 nM and results are shown (as percent inhibition compared to control) in Table 5.

Table 5

Human PP1γ1 Antisense Oligonucleotides

| Isis # | Sequence (5' 6 3') | Target source and site (Genbank #; nucleotide #s) | Target region | Percent Inhibition | SEQ ID NO: |
|---|---|---|---|---|---|
| 14430 | **CCATC**GCCTTCCCAC**CGCCG** | x74008; 139-158 | AUG | 26% | 26 |
| 14431 | **CATATT**TTGAGTGGT**GCTTC** | x74008; 320-339 | coding | 0 | 27 |
| 14432 | **TGGCA**CATTCATGGTT**CCCT** | x74008; 520-539 | coding | 32 | 28 |
| 14433 | **CTCCA**TGACAGCAGA**ATATC** | x74008; 658-677 | coding | 15 | 29 |
| 14434 | **GCAA**TAATTGGGCGC**AGAAA** | x74008; 954-973 | coding | 48 | 30 |
| 14435 | **GCTTG**CTTTGTGATC**ATACC** | x74008; 1097-1116 | coding | **91** | 31 |
| 14436 | **GATTC**AGAGCACCCT**AGGGC** | x74008; 1497-1516 | 3' UTR | **73** | 32 |
| 14437 | **AGTGA**TGCTGGCAAG**GTTGA** | x74008; 1671-1690 | 3' UTR | 37 | 33 |
| 14438 | **CCCAA**GAAGGCAGCA**TGTGT** | x74008; 1848-1867 | 3' UTR | 16 | 34 |
| 14439 | **AATGG**ACGGGTTCAG**GCCTG** | x74008; 2004-2023 | 3' UTR | **54** | 35 |
| 14440 | **AAAGC**ATAATCGGTC**ACTCG** | x74008; 2062-2081 | 3' UTR | 31 | 36 |
| 14441 | **CACGG**TATTGTACAC**GGTCA** | x74008; 2234-2253 | 3' UTR | **55** | 37 |

In this initial screen, oligonucleotides giving a reduction of PP1γ1 mRNA of approximately 50% or greater were considered active. According to this criterion, oligonucleotides 14435, 14436, 14439 and 14441 were found to be active. These sequences (SEQ ID NO: 31, 32, 35 and 37, respectively, SEQ ID NOs shown in **bold** in Table 5) are

therefore preferred. Of these, oligonucleotides 14435 and 14436 (SEQ ID NO: 31 and 32, respectively) showed at least 70% inhibition of PP1γ1 mRNA in this assay and are highly active.

Dose response curves were obtained for several PP1γ1 oligonucleotides, from which $IC_{50}$s were calculated. ISIS 14439 demonstrated an $IC_{50}$ of less than 50 nM. ISIS 14441 demonstrated an $IC_{50}$ of approximately 125 nM. ISIS 14436 showed an $IC_{50}$ of approximately 150 nM and ISIS 14435 showed an $IC_{50}$ of approximately 180 nM. ISIS 15032, an analog of ISIS 14435 with a uniformly phosphorothioate backbone and 2' MOE wings flanking a 10-nucleotide deoxy gap, demonstrated an estimated $IC_{50}$ of between 50 and 100 nM.

**Additional oligonucleotides to PP5**

The oligonucleotides shown in Table 6 were designed using the Genbank sequence HSRNAPPP5 (Genbank accession number X89416.1 (SEQ ID NO: 43), synthesized as described herein, and tested for inhibition of PP5 mRNA expression in A549 cells using a Northern blot assay. All oligonucleotides shown in Table 6 are chimeric oligonucleotides with central 10 bp 2'-deoxy "gaps," which have phosphorothioate backbones, flanked on both sides by 5 bp 2'-methoxyethoxy (2'-MOE) "wings" (shown in **bold**), which have phosphodiester backbones. All cytosines in the 2'-MOE wings are 5-methylcytosines.

Table 6

Additional human PP5 Antisense Oligonucleotides

| Isis # | Sequence (5' 6 3') | Target source and site (Genbank #; nucleotide #s) | Target region | Percent Inhibition | SEQ ID NO: |
|---|---|---|---|---|---|
| 134202 | **TCTTG**AGCTCCTCTG**CCCGC** | X89416; nt 61-80; | Coding | 35 | 44 |
| 134203 | **GGCGT**TCTCGTAGTC**CTTGG** | X89416; nt 105-124; | coding | 38 | 45 |
| 134204 | **CTGCG**GTTGCCATAG**TAGAT** | X89416; nt 170-189; | coding | N.D. | 46 |
| 134205 | **CACTC**AGTGCGCAGG**TAGGC** | X89416; nt 194-203; | coding | 38 | 47 |
| 134206 | **CAGCT**CAATGGCCCG**CGTGG** | X89416; nt 237-256; | coding | 37 | 48 |
| 134207 | **CAGCG**CGGCCCGGAA**CTTGC** | x89416; nt 312-331; | coding | 36 | 49 |
| 134208 | **ACGAT**CTTGTTGCAC**TCCTG** | x89416; nt 392-411; | coding | 37 | 50 |
| 134209 | **CGCGA**TGGCCCGCTC**AAAGG** | x89416; nt 423-442; | coding | 37 | 51 |
| 134210 | **TGTCC**AGCGAGTCCA**CCACG** | x89416; nt 463-482; | coding | 2 | 52 |
| 134211 | **GTCTT**CAAGCTTGGG**TCCGC** | x89416; nt 513-532; | coding | 28 | 53 |
| 134212 | **CATTT**CCGGTGCAGT**TTCTT** | x89416; nt 587-606; | coding | 23 | 54 |
| 134213 | **GAGGT**CATAGAACTG**GCCAT** | x89416; nt 714-733; | coding | 33 | 55 |
| 134214 | **GTCTC**CGAGGGTAAA**CCGTT** | x89416; nt 752-771; | coding | 35 | 56 |
| 134215 | **AAGGA**GCCTCGGTCC**ACAAA** | x89416; nt 797-816; | coding | 34 | 57 |
| 134216 | **TCTGT**CTCGTGGTTG**CCTCG** | x89416; nt 884-903; | coding | 36 | 58 |
| 134217 | **TGGCC**TTCACCTCAC**CCTCG** | x89416; nt 928-947; | coding | 92 | 59 |
| 134218 | **GCCAA**CGGGAGCCAC**TCGAA** | x89416; nt 986-1005; | coding | 96 | 60 |
| 134219 | **GGGTT**GTCGATTCCG**CTCAA** | x89416; nt 1089-1108; | coding | 88 | 61 |
| 134220 | **CCCGT**TCTGTGGCTG**TGGAT** | x89416; nt 1146-1165; | coding | 90 | 62 |

| | | | | | |
|---|---|---|---|---|---|
| 134221 | **AGAAG**GCCTTGGTGA**CGTCA** | x89416; nt 1207-1226; | coding | N.D. | 63 |
| 134222 | **ACGGT**GACACAGCGG**CCTCC** | x89416; nt 1298-1317; | coding | 68 | 64 |
| 134223 | **GTGGA**ACTGAGGCCG**TAGGT** | x89416; nt 1386-1405; | coding | 82 | 65 |
| 134224 | **AGGCC**ATGGGCTTGA**CGTTG** | x89416; nt 1429-1448; | coding | 98 | 66 |
| 134225 | **CCGCC**CCGCCCGTCA**CCTCA** | x89416; nt 1481-1500; | coding | N.D. | 67 |
| 134226 | **CAGGG**CCTGGGTCCG**GTGGG** | x89416; nt 1525-1544; | 3'-UTR | 77 | 68 |
| 134227 | **CCCTA**CCCCCTCTGC**AAACC** | x89416; nt 1626-1645; | 3'-UTR | N.D. | 69 |
| 134228 | **AGACC**CTCTGGCCAG**CCCCT** | x89416; nt 1654-1663 | 3'-UTR | 87 | 70 |
| 134229 | **ACTCG**GCCCCACCCC**ACCCC** | x89416; nt 1754-1773 | 3'-UTR | N.D. | 71 |
| 134230 | **GAGCC**CCAGCCTAGC**CCCAC** | x89416; nt 1903-1922 | 3'-UTR | 63 | 72 |

N.D.=not determined. In this initial screen, oligonucleotides giving a reduction of PP5 mRNA of approximately 50% or greater were considered active. According to this criterion, oligonucleotides 134217-134220, 134222-134224, 134226, 134228 and 134230 were found to be active. These sequences (SEQ ID NO: 59-62, 64-66, 68, 70 and 72, respectively) and are therefore preferred. Of these, oligonucleotides 134217-134220, 134223, 134224, 134226 and 134228 (SEQ ID NO: 59-62, 65, 66, 68 and 70, respectively) showed at least 70% inhibition of PP5 mRNA in this assay and are highly active.

**Specificity of oligonucleotides for PP isoforms:**

Several oligonucleotides which were determined to be active against their particular target protein phosphatase isoform were tested to see if they had any effects on other isoforms. None of the PP1γ oligonucleotides tested (14430, 14431, 14432, 14433, 14434 and 14435) had any effect on PP2Aα mRNA levels. ISIS 14441 was also tested for ability

to inhibit PP5 and had no effect. ISIS 14439, another oligonucleotide targeted to PP1γ1, had no effects on the other PP1 isoforms, PP1α and PP1β. ISIS 15032, the uniformly P=S analog of 14435, also had no inhibitory effect on PP1α or PP1β mRNA levels.

**EXAMPLES**

**Example 1**

**Synthesis and Characterization of Oligonucleotides**

Unmodified oligodeoxynucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine. â-cyanoethyldiisopropyl-phosphoramidites are purchased from Applied Biosystems (Foster City, CA). For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by a 0.2 M solution of $^{3}$H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation cycle wait step was increased to 68 seconds and was followed by the capping step.

2'-methoxy oligonucleotides were synthesized using 2'-methoxy β-cyanoethyldiisopropyl-phosphoramidites (Chemgenes, Needham MA) and the standard cycle for unmodified oligonucleotides, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds. Other 2'-alkoxy oligonucleotides were synthesized by a modification of this method, using appropriate 2'-modified amidites such as those available from Glen Research, Inc., Sterling, VA.

2'-fluoro oligonucleotides were synthesized as described in Kawasaki et al., *J. Med. Chem.* **1993**, 36, 831-841. Briefly, the protected nucleoside N$^{6}$-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially

available 9-ß-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-α-fluoro atom is introduced by a $S_N2$-displacement of a 2'-ß-O-trifyl group. Thus $N^6$-benzoyl-9-ß-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and $N^6$-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl- (DMT) and 5'-DMT-3'-phosphoramidite intermediates.

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-ß-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2, 2'-anhydro-1-ß-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give $N^4$-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-(2-methoxyethyl)-modified amidites are synthesized according to Martin, P., *Helv. Chim. Acta* **1995**, 78,486-504. For ease of synthesis, the last nucleotide was a deoxynucleotide. 2'-O-$CH_2CH_2OCH_3$-cytosines may be 5-methyl cytosines.

*Synthesis of 5-Methyl cytosine monomers:*

2,2'-Anhydro[1-(β-D-arabinofuranosyl)-5-methyluridine]:

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid which was crushed to a light tan powder (57 g, 85% crude yield). The material was used as is for further reactions.

2'-O-Methoxyethyl-5-methyluridine:

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1

L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in $CH_3CN$ (600 mL) and evaporated. A silica gel column (3 kg) was packed in $CH_2Cl_2$/acetone/MeOH (20:5:3) containing 0.5% $Et_3NH$. The residue was dissolved in $CH_2Cl_2$ (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with $CH_3CN$ (200 mL). The residue was dissolved in $CHCl_3$ (1.5 L) and extracted with 2x500 mL of saturated $NaHCO_3$ and 2x500 mL of saturated NaCl. The organic phase was dried over $Na_2SO_4$, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/Hexane/Acetone (5:5:1) containing 0.5% $Et_3NH$. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyl-uridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in $CHCl_3$ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of $CHCl_3$. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/Hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine:

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in $CH_3CN$ (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in $CH_3CN$ (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. $POCl_3$ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the later solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the

reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of $NaHCO_3$ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine:

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and $NH_4OH$ (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with $NH_3$ gas was added and the vessel heated to 100°C for 2 hours (tlc showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

$N^4$-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, tlc showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in $CHCl_3$ (700 mL) and extracted with saturated $NaHCO_3$ (2x300 mL) and saturated NaCl (2x300 mL), dried over $MgSO_4$ and evaporated to give a residue (96

g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (1:1) containing 0.5% $Et_3NH$ as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

$N^4$-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite:

$N^4$-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in $CH_2Cl_2$ (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction mixture was extracted with saturated $NaHCO_3$ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with $CH_2Cl_2$ (300 mL), and the extracts were combined, dried over $MgSO_4$ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc\Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

5-methyl-2'-deoxycytidine (5-me-C) containing oligonucleotides were synthesized according to published methods (Sanghvi et al., 1993, Nucl. Acids Res. 21:3197-3203) using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

Oligonucleotides having methylene(methylimino) backbones are synthesized according to U.S. Patent 5,378,825, which is coassigned to the assignee of the present invention and is incorporated herein in its entirety. Other nitrogen-containing backbones are synthesized according to WO 92/20823 which is also

coassigned to the assignee of the present invention and incorporated herein in its entirety.

Oligonucleotides having amide backbones are synthesized according to De Mesmaeker et al., Acc. Chem. Res. 1995, 28, 366-374. The amide moiety is readily accessible by simple and well-known synthetic methods and is compatible with the conditions required for solid phase synthesis of oligonucleotides.

Oligonucleotides with morpholino backbones are synthesized according to U.S. Patent 5,034,506 (Summerton and Weller).

Peptide-nucleic acid (PNA) oligomers are synthesized according to P.E. Nielsen et al., *Science* **1991**, 254, 1497).

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by $^{31}P$ nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., 1991, J. Biol. Chem., 266:18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

**Example 2**

**Northern blot analysis of inhibition of protein phosphatase mRNA expression**

The human lung tumor cell line A549 was obtained from the American Type Culture Collection (Rockville MD)and were

grown in DMEM (Gibco BRL, Gaithersburg MD), supplemented with 10% fetal calf. Cells were seeded on 60 mm plates. When they reached 70% confluency, they were washed with DMEM and 1 ml of DMEM containing 15 ìg/ml DOTMA/DOPE (Lipofectin®, GIBCO-BRL) and oligonucleotide at desired concentration was added. Duplicate dishes were used for each treatment condition. After 4 hours of treatment at 37°C, cells were washed and cultured in fresh DMEM containing 10% fetal bovine serum for an additional 17 hours. Cells were then harvested and total RNA was extracted with TRIzol Reagent (GIBCO-BRL) according to manufacturer's protocol. Total RNA (20 µg) was fractionated on a 1% agarose gel containing formaldehyde, and transferred to a DURLON-UV (Stratagene) nylon membrane. Following UV crosslinking, the filters were hybridized with the appropriate protein phosphatase probe. The $^{32}$P-labeled human PP cDNA probes are generated with DECAPrime$^{R}$ DNA Labeling Kit (Ambion) according to the manufacturer's protocol. Hybridization was performed in a hybridization solution containing 50% formamide at 42°C for 16 hours. This was followed by two low stringency washes (2x SSC) at room temperature and two high stringency washes (0.1x SSC/0.5% SDS) at 55°C. Hybridization signals were visualized by autoradiography, and filters were then stripped and reprobed with a $^{32}$P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to confirm equal loading. The densities of hybridization signals were measured with the NIH Image program (ImagePC).

**Example 3**

**Antisense inhibition of cell proliferation**

A549 cells were seeded in 12-well tissue culture plates at about 50% confluence in DMEM containing 10% fetal bovine serum. The next day, cells were treated with the PP5-

specific antisense oligonucleotide, ISIS 15534, or its scrambled control, ISIS 15521, at a final concentration of 300 nM as described in Example 2. On each of the following 5 days, cultures from three wells of each treatment gruop were trypsinized, collected and counted. Cell viability was determined by trypan blue staining, and the results given are the mean of three independent experiments.

**Example 4**

**Measurement of [$^3$H]-thymine incorporation:**

A549 cells were subcultured in 24-well tissue culture plates and treated with the PP5-specific antisense oligonucleotide ISIS 15534 or its scrambled control, ISIS 15521 at a final concentration of 300 nM as described in Example 2. At timed intervals during the next 5 days, cells were pulse-labeled with [$^3$H]-thymidine (0.5 μCi/ml) for 5 hours. The cells were then lysed, and [$^3$H]-thymidine incorporation was determined by liquid scintillation counting using standard methods (Baserga, R. and Ashihara, T., (1979) Methods in Enzymology LVIII:248-262). The results given are the mean of three independent experiments.

**Example 5**

**Effect of oligonucleotides on calcium channels**

The effect of antisense oligonucleotides on calcium channels was tested in RINm5f cells. This is an insulin-secreting insulinoma rat cell line available from the American Type Culture Collection, Rockville MD. Calcium currents were measured using standard patch-clamp techniques to measure ion conductance. These techniques are described in, for example, Ammala et al., 1994, Proc. Natl. Acad. Sci., USA, 91:4343-4347.

**Example 6**

**A549 xenografts**

A549 cells are obtained from the American Type Culture Collection (Bethesda MD) and grown in T-75 flasks until 65-75% confluent. 5 x $10^6$ A549 cells are implanted subcutaneously in the inner thigh of nude mice. The PP5-specific antisense oligonucleotide, ISIS 15534, or its scrambled control, ISIS 15521, suspended in saline, are administered once daily by intravenous injection at doses ranging from 0.006 to 6.0 mg/kg. Resulting tumors are measured on days 9, 12, 17 and 21 and tumor volumes are calculated.

**Example 7**

**Detection of Protein Phosphatase Expression**

PP-specific oligonucleotides are radiolabeled after synthesis by $^{32}P$ labeling at the 5' end with polynucleotide kinase. Sambrook et al., *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989, Volume 2, pg. 11.31-11.32. Radiolabeled oligonucleotides are contacted with tissue or cell samples suspected of PP expression, such as tumor biopsy samples, under conditions in which specific hybridization can occur, and the sample is washed to remove unbound oligonucleotide. Radioactivity remaining in the sample indicates bound oligonucleotide and is quantitated using a scintillation counter or other routine means.

Analogous assays for fluorescent detection of PP expression use oligonucleotides of the invention which are labeled with fluorescein or other fluorescent tags. Labeled DNA oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine. ß-cyanoethyldiisopropyl phosphoramidites are

purchased from Applied Biosystems (Foster City, CA). Fluorescein-labeled amidites are purchased from Glen Research (Sterling VA). Incubation of oligonucleotide and biological sample is carried out as described for radiolabeled oligonucleotides except that instead of a scintillation counter, a fluorimeter or fluorescence microscope is used to detect the fluorescence which indicates PP expression.

**Example 8**

**Design and screening of duplexed antisense compounds targeting human serine/threonine protein phosphatases**

In accordance with the present invention, a series of nucleic acid duplexes comprising the antisense compounds of the present invention and their complements can be designed to target human serine/threonine protein phosphatases. The nucleobase sequence of the antisense strand of the duplex comprises at least a portion of an oligonucleotide to a human serine/threonine protein phosphatase as described herein. The ends of the strands may be modified by the addition of one or more natural or modified nucleobases to form an overhang. The sense strand of the dsRNA is then designed and synthesized as the complement of the antisense strand and may also contain modifications or additions to either terminus. For example, in one embodiment, both strands of the dsRNA duplex would be complementary over the central nucleobases, each having overhangs at one or both termini. For example, a duplex comprising an antisense strand having the sequence CGAGAGGCGGACGGGACCG and having a two-nucleobase overhang of deoxythymidine(dT) would have the following structure:

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              cgagaggcggacgggaccgTT    Antisense Strand
              ||||||||||||||||||||
            TTgctctccgcctgccctggc      Complement
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RNA strands of the duplex can be synthesized by methods disclosed herein or purchased from Dharmacon Research Inc., (Lafayette, CO). Once synthesized, the complementary strands are annealed. The single strands are aliquoted and diluted to a concentration of 50 uM. Once diluted, 30 uL of each strand is combined with 15uL of a 5X solution of annealing buffer. The final concentration of said buffer is 100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, and 2mM magnesium acetate. The final volume is 75 uL. This solution is incubated for 1 minute at 90°C and then centrifuged for 15 seconds. The tube is allowed to sit for 1 hour at 37°C at which time the dsRNA duplexes are used in experimentation. The final concentration of the dsRNA duplex is 20 uM. This solution can be stored frozen (-20°C) and freeze-thawed up to 5 times.

Once prepared, the duplexed antisense compounds are evaluated for their ability to modulate human serine/threonine protein phosphatase expression according to the protocols described herein.

**Example 9**

**Design of phenotypic assays and *in vivo* studies for the use of human serine/threonine protein phosphatase inhibitors**

Phenotypic assays

Once human serine/threonine protein phosphatase inhibitors have been identified by the methods disclosed herein, the compounds are further investigated in one or more phenotypic assays, each having measurable endpoints predictive of efficacy in the treatment of a particular disease state or condition. Phenotypic assays, kits and reagents for their use are well known to those skilled in the art and are herein used to investigate the role and/or association of human serine/threonine protein phosphatase in health and disease. Representative phenotypic assays,

which can be purchased from any one of several commercial vendors, include those for determining cell viability, cytotoxicity, proliferation or cell survival (Molecular Probes, Eugene, OR; PerkinElmer, Boston, MA), protein-based assays including enzymatic assays (Panvera, LLC, Madison, WI; BD Biosciences, Franklin Lakes, NJ; Oncogene Research Products, San Diego, CA), cell regulation, signal transduction, inflammation, oxidative processes and apoptosis (Assay Designs Inc., Ann Arbor, MI), triglyceride accumulation (Sigma-Aldrich, St. Louis, MO), angiogenesis assays, tube formation assays, cytokine and hormone assays and metabolic assays (Chemicon International Inc., Temecula, CA; Amersham Biosciences, Piscataway, NJ).

In one non-limiting example, cells determined to be appropriate for a particular phenotypic assay (i.e., MCF-7 cells selected for breast cancer studies; adipocytes for obesity studies) are treated with human serine/threonine protein phosphatase inhibitors identified from the *in vitro* studies as well as control compounds at optimal concentrations which are determined by the methods described above. At the end of the treatment period, treated and untreated cells are analyzed by one or more methods specific for the assay to determine phenotypic outcomes and endpoints.

Phenotypic endpoints include changes in cell morphology over time or treatment dose as well as changes in levels of cellular components such as proteins, lipids, nucleic acids, hormones, saccharides or metals. Measurements of cellular status which include pH, stage of the cell cycle, intake or excretion of biological indicators by the cell, are also endpoints of interest.

Analysis of the genotype of the cell (measurement of the expression of one or more of the genes of the cell) after treatment is also used as an indicator of the

efficacy or potency of the human serine/threonine protein phosphatase inhibitors. Hallmark genes, or those genes suspected to be associated with a specific disease state, condition, or phenotype, are measured in both treated and untreated cells.